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*Dictyostelium discoideum*의 분화에서 glutathione의 역할

Roles of glutathione in differentiation of *Dictyostelium discoideum*

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Roles of glutathione in differentiation of *Dictyostelium discoideum*

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ABSTRACT

Reduced glutathione (GSH, γ-L-glutamyl-L-cysteinylglycine) is a ubiquitous tripeptide found in almost all organisms and the most abundant non-protein thiol-containing compound in eukaryotic cells. It is known to participate in diverse cellular functions, such as antioxidant defenses, the regulation of intracellular redox status, signal transduction, cell proliferation and death, and immune responses. GSH also participates in regulation of organ differentiation.

Previously, it was reported that GSH serves important roles in normal growth and differentiation of *Dictyostelium discoideum*. The developmental morphology of g*csA*$^{-}$ cells was dependent on the concentration of GSH which was added to culture media. In this work, to find out the precise roles of GSH during development, intracellular GSH was completely depleted and then developmental morphology was observed. Absence of GSH caused defects in the formation of multicellular aggregates. g*csA*$^{-}$ cells were in a state of single cells if GSH was not supplemented. This developmental defect of g*csA*$^{-}$ cells was rescued by adding exogenous GSH, γ-GC, or GSSG. But other thiol-compounds or antioxidant molecules, such as DTT, NAC, and ascorbic acid, did not compensate GSH. These results indicate that GSH itself plays essential roles rather than as an antioxidant molecule in regulating the development of *Dictyostelium*.

To gain more information on the developmental defect of *gcsA*$^{-}$ cells, the expression patterns of genes that were required to initiate development were examined. GSH-depleted *gcsA*$^{-}$ cells failed to decrease the expression of a growth-stage-specific gene (*cprD*) and failed to induce the expression of genes

that encode proteins required for early development (discoidin, *dscA*; differentiation associated protein, *dia2*; cAMP receptor, *carA*/cAR1; adenylyl cyclase, *acaA*/ACA; and the catalytic subunit of protein kinase A, *pkaC*/PKA-C). Decreased expression of *carA* and *acaA* was remarkable in *gcsA*$^{-}$ cells. However, the developmental defect of *gcsA*$^{-}$ cells was not restored by cAMP stimulation or by cAR1 expression. Though constitutively expressed cAR1 induced the expression of *acaA* and Gα2 gene, *gcsA*$^{-}$ cells did not develop without GSH. These results suggest that GSH seems to work at higher step to the cAMP signaling pathway to regulate development of *Dictyostelium*.

YakA signaling is known the earliest response to environmental signal to initiate development and functions prior to cAMP signaling. The expression of *yakA* is responsible to induce the expression of differentiation-associated genes and to inhibit the expression of growth-phase genes for the initiation of development. The expression of *yakA* was regulated by intracellular GSH in both KAx3 and *gcsA*$^{-}$ cells. GSH-depleted *gcsA*$^{-}$ cells showed undetectably low *yakA* expression levels, but the expression was induced by adding GSH. The expression of *yakA* was in proportion to the concentration of exogenously added GSH in KAx3 cell. Further, induced *yakA* expression promoted the formation of multicellular aggregate in both KAx3 and *gcsA*$^{-}$ cells. Intracellular GSH also influenced on the expression of *pufA* and the activity of PKA, which are components of downstream regulators in the YakA signaling pathway. *gcsA*$^{-}$ cells showed increased *pufA* expression and lowered PKA activity compared to KAx3 cells. However, the expression of *pufA* and the activity of PKA were recovered to the similar level of KAx3 cells by adding GSH. Interestingly, *yakA*$^{-}$ cells showed similar gene expression pattern and developmental morphology to *gcsA*$^{-}$ cells. *yakA*$^{-}$ cells did not develop. The expression of *carA*

and *acaA* was significantly decreased and the activity of PKA was not detected in *yakA*$^{-}$ cells. Exogenous GSH did not rescue the developmental defects of *yakA*$^{-}$ cells, but constitutively expressed YakA in *gcsA*$^{-}$ cells (YakA^{OE}/*gcsA*$^{-}$) rescued the developmental defects of *gcsA*$^{-}$ cells without the addition of GSH; YakA^{OE}/*gcsA*$^{-}$ cells formed multicellular aggregates and *carA* and *acaA* were expressed without GSH. These results indicate that intracellular GSH plays indispensable roles during development by regulating the expression of *yakA* in *Dictyostelium*.

To investigate the relation between YakA and GSH further, the concentration of intracellular GSH the expression of *gcsA* were monitored in *yakA*$^{-}$ and YakA^{OE}/KAx3 cells. *yakA*$^{-}$ cells showed decreased intracellular GSH levels around 40% compared to KAx3 and considerably increased *gcsA* expression. However, constitutive expression of YakA in KAx3 cells (YakA^{OE}/KAx3 cells) did not significantly influence on the intracellular GSH level and *gcsA* expression, indicating that GSH regulates the expression of *yakA* but YakA did not regulate intracellular GSH. Decreased intracellular GSH concentration might be caused by hypersensitiveness to oxidative stress of *yakA*$^{-}$ cells and leads to accumulation of *gcsA* transcripts by the feedback regulation of GSH.

Taken together, these findings suggest that GSH plays an essential role in the transition from growth to differentiation by modulating the expression of the genes encoding YakA as well as components that act downstream in the YakA signaling pathway in *Dictyostelium*.

Key words: Glutathione; YakA; Transition from growth to differentiation; *Dictyostelium discoideum*

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LIST OF ABBREVIATIONS

GSH	reduced glutathione
GSSG	oxidized glutathione
γ-GC	γ-glutamylcysteine
GCS	γ-glutamylcysteine synthetase
GSS	glutathione synthetase
ROS	reactive oxygen species
PSF	prestarvation factor
CMF	conditioned medium factor
cAMP	3′-5′-cyclic adenosine monophosphate
cAR1	cAMP receptor
ACA	adenylyl cyclase
PKA	cAMP-dependent protein kinase A
bp	base pair
Da	dalton
rpm	revolutions per minute
RT	reverse transcriptase
PCR	polymerase chain reaction
HPLC	high performance liquid chromatography
LB	Luria-Bertani
DTT	dithiothreitol
NAC	*N*-acetylcysteine
mBBr	monobromobimane
NEM	*N*-ethylmaleimide
SDS	sodium dodecyl sulfate
EDTA	ethylenediaminetetraacetate
DEPC	diethylpyrocarbonate

I. INTRODUCTION

1. Glutathione

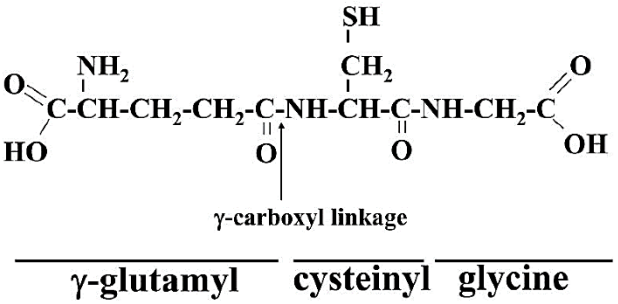
1.1. An overview

Glutathione (GSH) is a ubiquitous tripeptide, γ-L-glutamyl-L-cysteinylglycine (Scheme 1A), found in most plants, microorganisms, and all mammalian tissues (Meister and Anderson, 1983). It is the main derivative of cysteine and the most abundant intracellular non-protein thiol. Eukaryotic cells have three major reservoirs of GSH. Almost 90% of cellular GSH are in the cytosol, 10% in the mitochondria, and a small percentage in the endoplasmic reticulum (Meredith and Reed, 1982; Hwang *et al.*, 1992).

Glutathione exists in thiol-reduced form (GSH) and disulfide-oxidized (GSSG) form (Kaplowitz *et al.*, 1985). Under physiological conditions, most cellular constituents are reduced. In cells, glutathione is maintained in the reduced form (GSH) by the action of glutathione reductase and NAD(P)H. Because the oxidized form (GSSG) is efficiently reduced, the intracellular ratio of GSH to GSSG is high in most eukaryotic cells (Halliwell and Gutteridge, 1989). GSSG content is rarely exceeds 10% of total glutathione (Akerboom *et al.*, 1982; Halliwell and Gutteridge, 1989; Wu *et al.*, 2004). Maintaining optimal GSH:GSSG ratios in cells is critical to survival, hence, tight regulation of the system is imperative. The GSH to GSSG ratio is often used as an indicator of the cellular redox state.

The potent electron donating capacity of sulfhydryl group is the key to the multiple actions of GSH at the molecular, cellular and tissue level (Meister, 1994). The free sulfhydryl moiety of the cysteine residue confers high redox potential $E'_0 = -0.33$ V (Lewin, 1976). Its high negative redox potential renders GSH both a potent antioxidant and a convenient cofactor

A



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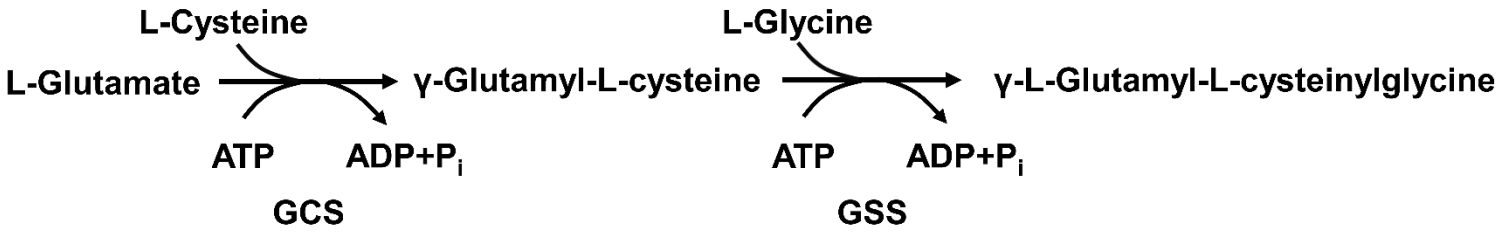


Scheme 1. Chemical structure and enzymatic synthesis of GSH (γ-L-Glutamyl-L-cysteinylglycine).

(A) Chemical structure of GSH. (B) Enzymatic biosynthesis of GSH. GCS; γ-glutamylcysteine synthetase, GSS; glutathione synthetase.

for enzymatic reactions that require readily available electron pairs. Intracellular stability, which is promoted by the exceptional γ-glutamyl linkage and lack of the toxicity associated with cysteine (Vina *et al.*, 1983), make GSH suitable as a cellular thiol redox buffer to maintain a thiol/disulfide redox potential.

In cells, tissues, and plasma, glutathione is present in several additional forms. Glutathione disulfide (GSSG) is formed upon oxidation. Other forms of disulfide are of the mixed type, GSSR, a major class of biologically interesting ones being glutathione-cysteinyl disulfides on proteins.

1.2. The enzymatic synthesis of glutathione

The synthesis of GSH from glutamate, cysteine, and glycine is catalyzed sequentially by two cytosolic enzymes, γ-glutamylcysteine synthetase (GCS) and GSH synthetase (GSS) (Scheme 1B).

In the GCS reaction, the γ-carboxyl group of glutamate reacts with the amino group of cysteine to form a peptide γ-linkage, which protects GSH from hydrolysis by intracellular peptidase. γ-Glutamylcysteine synthetase is rate-limiting enzyme in de novo synthesis of GSH (Meister, 1983). Induction of GCS expression has been demonstrated in response to diverse stimuli in a cell specific manner. The bioavailability of cysteine regulates the synthesis of GSH. Post-translational modification of GCS also influence GSH synthesis (Bella *et al.*, 1999; Gomi *et al.*, 1997). Specifically, phosphorylation of GCS leads to the inhibition of GSH synthesis. GSH itself regulates the activity of GCS via a negative feedback mechanism (Meister and Anderson, 1983). Hence, GSH depletion increases the rate of GSH

synthesis. The mechanistic links between feedback inhibition and thiol/disulfide redox regulation of GCS remain to be elucidated.

Mammalian GCS is a heterodimer consisting of a catalytically active heavy subunit (GCS_h, 73 kDa) and a light regulatory subunit (GCS_l, 31 kDa) (Lu, 2000). There is a variable degree of sequence identity among the cDNA sequences and the deduced amino acid sequences of the various eukaryotic GCS catalytic subunit proteins. The cDNA of mammalian and yeast cDNA sequences shows the highest degree of similarity (90-95%) (Griffith and Mulcahy, 1999). By contrast, the bacterial and Arabidopsis GCS encode catalytic proteins that are smaller than those of the other species examined and share only limited amino acid sequence identity (<10% and <20%, respectively) (Griffith and Mulcahy, 1999).

GCS from *Dictyostelium discoideum* is distinct from the general eukaryotic forms. It is a monomer with a subunit molecular mass of 75 kDa, encoded by a single gene. However, the deduced amino acid sequence of *Dictyostelium* GCS has considerable sequence similarity with the protein of *Drosophila melanogaster*, *Homo sapiens*, and *Schizosaccharomyces pombe*, approximately 48%, 47%, and 43%, respectively. Like other catalytic subunit of GCS, the catalytic center of the active site, Cys-256, and a common motif found in phosphate binding sites, glycine-rich loop (Gly-249, Gly-251, and Gly-253), are well conserved in *Dictyostelium* (Griffith and Mulcahy, 1999; Saraste *et al.*, 1990).

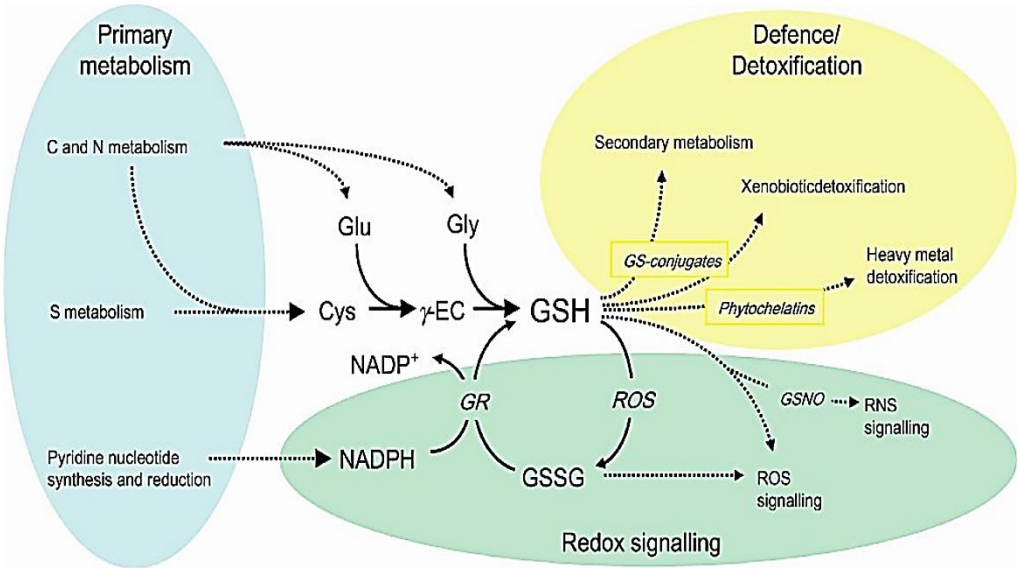
The second enzyme required for GSH biosynthesis is glutathione synthetase (GSS) (Scheme 1B). This enzyme functions as a homodimer of 118 kDa and is responsible for the addition of glycine to γ-glutamylcysteine created by GCS to form GSH. GSS is not subject to feedback inhibition by

GSH. In *Saccharomyces cerevisiae*, this enzyme is not essential for growth under both normal and oxidative stress conditions due to an accumulation of γ-glutamylcysteine, which protects against oxidative stress (Grant *et al.*, 1997). Overexpression of GSS failed to increase GSH level whereas overexpression of GCS increased the GSH level, consistent with the fact that GCS is the rate-limiting enzyme of GSH synthesis (Grant *et al.*, 1997).

1.3. The roles of glutathione in cellular reactions

Glutathione participates in many cellular reactions (Scheme 2). GSH displays remarkable metabolic and regulatory versatility. GSH/GSSG is the most important redox couple and plays crucial roles in an antioxidant defense, nutrient metabolism, and the regulation of pathways essential for whole cellular homeostasis.

First of all, GSH effectively scavenges free radicals and other reactive oxygen species (ROS) directly, and indirectly through enzymatic reactions (Grant *et al.*, 1996; Fang *et al.*, 2002) as part of the antioxidant barrier that prevents excessive oxidation of sensitive cellular components. In the mitochondria, GSH is particularly important because there is no catalase. Mitochondrial GSH is critical in defending against both physiologically and pathologically generated oxidative stress (Garcia-Ruiz and Fernandez-Checa, 2006). In such reactions, GSH is oxidized to form GSSG, which is then reduced to GSH by the NADPH-dependent glutathione reductase. Glutathione deficiency contributes to oxidative stress, and may play a key role in aging and the pathogenesis of many diseases. Decreased cellular levels of GSH have been observed in a number of diseases such as diabetes cancer, and HIV infection in which increased oxidative stress has been



Scheme 2. An integrated overview of the most important glutathione functions. Cys, cysteine; γ-EC, γ-glutamylcysteine; GS-conjugates, glutathione S-conjugates; GSNO, S-nitrosoglutathione; Glu, glutamate; Gly, glycine; RNS, reactive nitrogen species; ROS, reactive oxygen species (Noctor *et al.*, 2012).

implicated as the pathogenic metabolism (Townsend *et al.*, 2003).

Second, GSH maintains the intracellular redox balance and the essential thiol status of proteins (Lu, 1999). GSH undergoes thiol-disulfide exchange in a reaction catalyzed by thiol-transferase. As mentioned above, cellular GSSG content is extremely low so that protein mixed disulfide formation is limited. The thiol-disulfide equilibrium within the cell is known to regulate a diverse number of metabolic processes including enzyme activity, transport activity, signal transduction, and gene expression via alteration of redox sensitive transcription factors (Hutter *et al.*, 1997; Lu, 1999; Townsend *et al.*, 2003).

Third, GSH participates in cell signaling through at least two mechanisms, protein S-glutathionylation and cysteine S-nitrosylation (Zhang and Forman, 2012). These modifications change the conformation, stability, or activity of the target proteins. The former is formed when GSH conjugates with reactive cysteine residues within proteins. GSH also interact with nitric oxide (NO) system via formation of S-nitroglutathione (GSNO) (Lindermayr *et al.*, 2010). GSH may also indirectly participate in the redox signaling by changing cellular redox homeostasis (Sies, 1999).

Fourth, GSH regulates cell growth, proliferation, and cell death. Recent evidence suggests that an increased GSH level is associated with an early proliferative response and is essential for the cell to enter the S phase (Chaudhuri *et al.*, 1997; Lu, 2009; Aw, 2003). GSH modulates cell death at both extremes, apoptosis and necrosis, by regulating redox state of specific thiol residues of proteins such as NFκB, stress kinases, and caspases, involved in cell death (Galter *et al.*, 1994; Garcia-Ruiz and Fernandez-Checa, 2007). GSH depletion occurs during apoptosis in many different cell types,

secondary to increased reactive oxygen species (ROS), enhanced GSH efflux, and decreased GCS activity (Hall, 1999; Madeo *et al.*, 1999; Franklin *et al.*, 2003; Baek *et al.*, 2004).

Fifth, GSH functions in detoxification of xenobiotics or their metabolites (Ketterer *et al.*, 1983, Meister, 1994, Hayes and McLellan, 1999). GSH conjugates electrophilic those toxic compounds enzymatically or spontaneously in reactions catalyzed by GSH-S-transferase (Meister, 1988). The formed conjugates are usually excreted from the cell.

1.4. The roles of glutathione in development

The changes in redox environment during differentiation is provided by Allen *et al.* (1985) in a study of a slime mold (*Physarum polycephalum*). A sequential change in the antioxidant profile is also observed upon providing a stimulus for differentiation. As differentiation proceeded, superoxide dismutase (SOD) activity increases by as much as 21-fold. This increase in SOD activity parallels the rate of differentiation. In contrast, GSH concentration decreases during differentiation by more than 80% in all cultures, regardless of the initial concentration. The rate of differentiation is inversely related to the initial GSH concentration and directly proportional to the SOD activity. In sea urchin eggs, fluctuation of cellular thiols during development is also noted (Kawamura, 1960). Thomas *et al*. (1991) reported dramatic decrease in glutathione level during the thermal yeast-to-mycelial induction and suggested the potential involvement of intracellular glutathione levels in regulation of the morphogenesis in *Candida albicans*.

Recent studies have suggested an important role for GSH in mammalian development *in vitro* and *in vivo*. The embryo is exposed to increased

oxidative stress that exceeds the antioxidant defenses, resulting in a decrease in the GSH:GSSG ratio during subsequent development (Dumollard *et al.*, 2007). Glutathione levels increase during maturation of oocytes and subsequently decrease by 90% during early embryo development in blastocysts, in comparison to concentrations in mitotic stage oocytes (Gardiner and Reed, 1994; Luberda, 2005). The mechanism for this may involve ATP-dependent synthesis during oocyte maturation, which is switched off after fertilization. Pharmacologically induced GSH deficiency by an inhibitor (buthionine sulfoximine, BSO) in newborn mammals such as rats and guinea pig leads to rapid multi-organ failure and death within a few days (Meister, 1994). The generation of a null mutation of the heavy subunit of γ-glutamylcysteine synthetase results in complete GSH deficiency and caused embryo lethality in the mouse resulted from apoptotic cell death (Shi *et al.*, 2000; Winkler *et al.*, 2011).

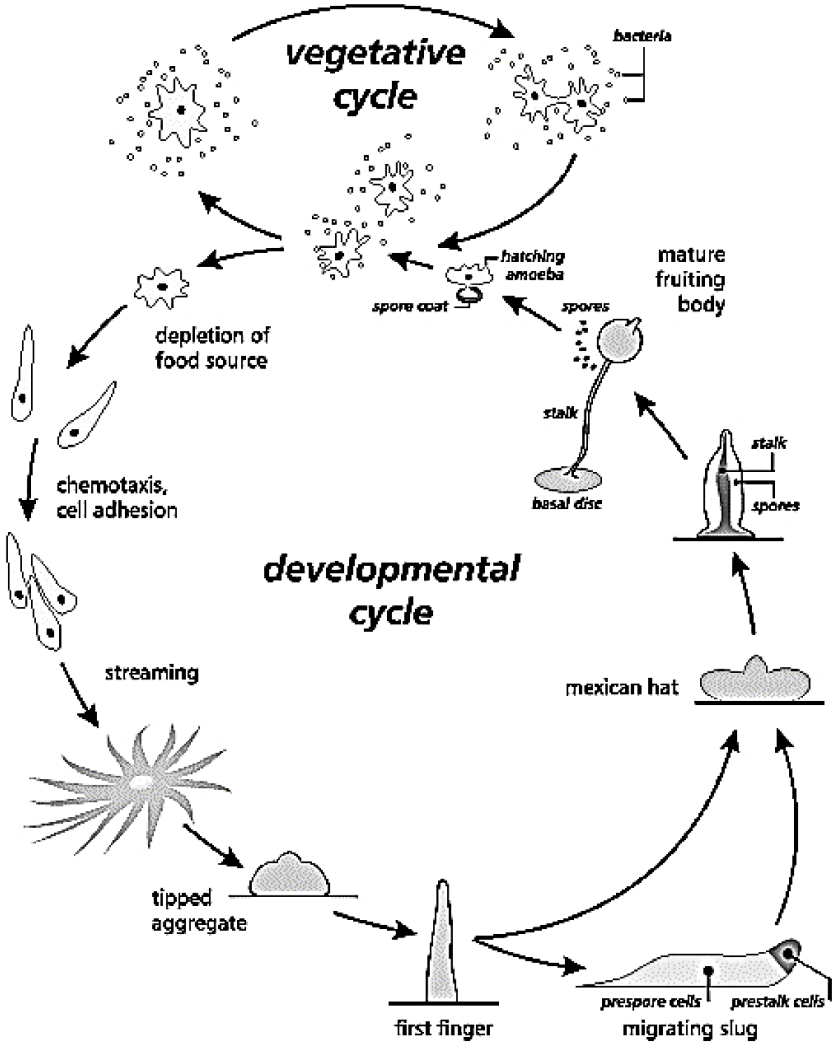
In plant cells, knocking out expression of GSH1, encoding the first enzyme of the committed pathway of GSH synthesis, causes lethality at the embryo stages (Cairns *et al.*, 2006; Noctor *et al.*, 2012), knockouts for GSH2, encoding glutathione synthetase, show a seedling-lethal phenotype (Pasternak *et al.*, 2008). In several mutants in which decreased GSH contents are caused by less severe mutation in the GSH1 gene. Of these mutants, which has less than 5% of wild-type glutathione contents, shows failure in development of a root apical meristem (Vernoux *et al.*, 2000). In other mutants, in which glutathione is decreased to about 25% to 50% of wild-type contents, developmental phenotypes are weak or absent, but alterations in environmental responses are observed.

2. *Dictyostelium discoideum*

2.1 Properties as a model organism

The cellular slime mold *Dictyostelium discoideum* is widely used to study multicellular morphogenesis. The life cycle of *Dictyostelium discoideum* comprises two phases. During the vegetative phase, cells grow as solitary amoebae and feed on bacteria in the soil and multiply by simple binary fission. As the food source becomes steadily depleted and the population increases, cells stop growing and initiate a coordinated developmental program that ultimately leads to the formation of a multicellular organism with only two main cell types, vacuolated stalk and dormant spore cells (Loomis, 1982; Firtel, 1995). Coordinated cell type differentiation and morphogenesis lead to a final fruiting body that allows the dispersal of spores which survive harsh environmental conditions (Scheme 3).

Upon starvation, cAMP is synthesized and released in nanomolar and a pulsatile manner from aggregation centers and attracts neighboring cells to migrate to the center. The pulsatile release of cAMP results in concentric or spiral-rings of amoebae which is called hemispherical mounds up to 100,000 cells that become enclosed in a protein cellulose slime sheath to form tight aggregates. A rise in the cAMP concentration to micro molar levels occurs (Abe and Yanagisawa, 1983), which initiates a developmental cascade (Schnitzler *et al.*, 1995). A protruding tip then forms at the apes of each aggregates. The tip behaves as an organizer, which are orchestrating all subsequent movements of developing cells (Raper, 1940; Durston, 1976; MacWilliams, 1982). Subsequently, once a tip has formed, the aggregate



Scheme 3. Life cycle of *Dictyostelium discoideum*. The life cycle of *Dictyostelium discoideum* consists of distinct two different phase. When nutrients are available, cells grow vegetatively as single-celled amoeba. When nutrients are deprived, cells initiate developmental life cycle (http://www.dictyostelium.com/).

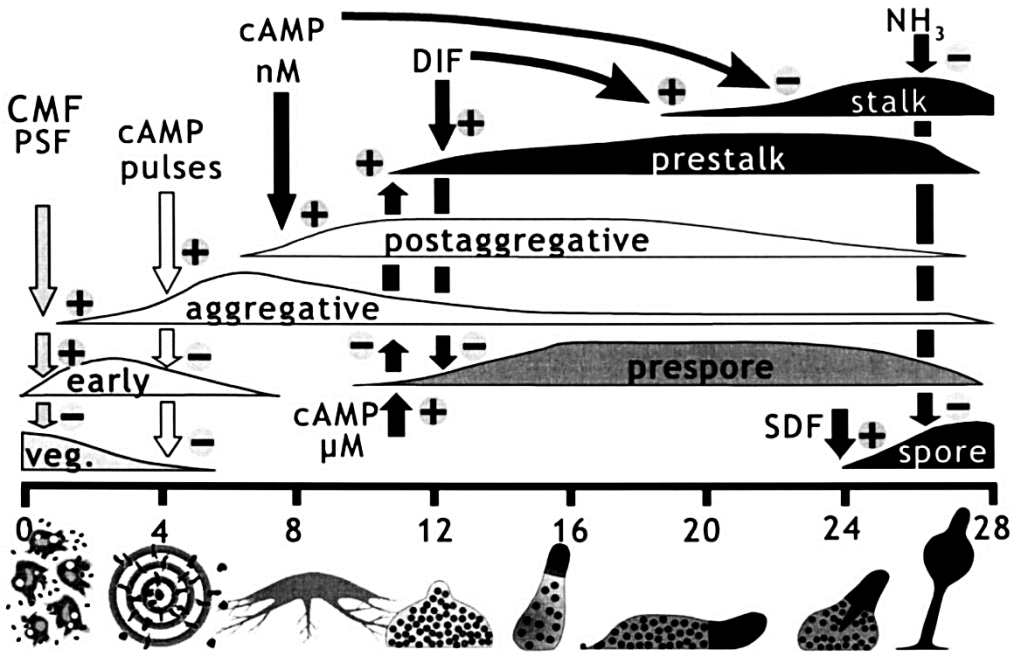
gradually elongates to give rise to an upright finger-like structure. In the finger and migrating slug, prestalk cells are localized to the anterior that are the precursors of the stalk cells of the mature fruiting body. Prespore cells are found in the posterior and the anterior-like cells (ALCs) which are scattered throughout slug (Gross, 1994). During culmination, prestalk cells penetrate through the prespore-cell mass and lift it off the substratum. Finally, cells mature into spore and stalk cells and form the final structure with a sorus atop a slender stalk (Loomis, 1982).

The life cycle of *Dictyostelium* cells is unique and relatively simple, but it contains almost all of the cellular processes such as cell movement, chemotaxis, cell adhesion, cell type determination, pattern formation, etc., essential for the establishment of multicellular organization. The 34 Mb genome contains many genes predicted to encode 125,000 proteins that are homologous to those in higher eukaryotes and are missing in *Saccharomyces cerevisiae*. Thus, *Dictyostelium* is a powerful system for genetic and functional analysis of gene function. The availability of biochemical and molecular genetics techniques has allowed the discovery of complex signaling networks which are important for *Dictyostelium* development and are also conserved in other organisms. The recent completion of the *Dictyostelium* genome sequence (Eichinger *et al.*, 2005; and accessible with related on-line resources at http://dcitybase.org) greatly facilitates such analysis. This relative simplicity has made *Dictyostelium* a model system for studying eukaryotic signal transduction and cell to cell communication during differentiation (Devreotes, 1994; Firtel, 1995; Williams, 1995).

2.2 The transition from growth to development

The process of transition from growth to development is of general importance for the development of organisms. Growth and differentiation are mutually exclusive, but they are cooperatively regulated during the course of development (Maeda, 2005). Similarly to most higher eukaryotic cells, the transition is regulated by complex molecular mechanisms, designed to ensure that development only occurs under optimal conditions in *Dictyostelium* (Scheme 4). Extracellular signals control the transition from growth to development and the changes are controlled by the activities of numerous regulators (William *et al.*, 1993; Katoh *et al.*, 2007). Cells prepare for future starvation and development by sensing environmental conditions and accumulating transcripts of a number of genes. Upon starvation, the expression of vegetative genes is reduced, whereas genes required for development are induced. When nutrients are depleted, cells stop replicating chromosomal DNA and reduce the expression of vegetative genes. However, the expression of genes required for development is induced to trigger aggregation.

Recent studies of gene transcription profiles show that aggregation of unicellular amoebae to multicellular structures is accompanied by a change in the expression of more than 25% of the genes in the genome (Van Driessche *et al.*, 2002). For example, the expression of V4 transcripts is induced by starvation. Antisense inhibition of V4 expression leads to a failure to inhibit the transcription of vegetative stage genes, and also leads to a reduction in the transcription of genes that are involved in the events of chemotaxis to cAMP (McPherson and Singleton, 1992). Among the genes that are repressed during early development, the transcription of several ribosomal protein genes has been rapidly reduced after starvation begins



Scheme 4. Regulation of gene expression during development of *Dictyostelium discoideum.* The transition from growth to development is regulated by modulating gene expression designed to ensure that aggregation occurs under optimal conditions. CMF, conditioned medium factor; PSF, prestarvation factor; DIF, differentiation inducing factor; SDF, spore differentiation factor; veg., vegetative. (Williams *et al.*, 1993)

(Ken and Singleton, 1994). The transcripts of several biosynthetic genes also disappear. These include transcripts for *cprD*, a growth stage cysteine proteinase (Souza et al., 1995), *pyr56*, UMP-synthetase, and *guaA*, GMP-synthetase (Jacquet *et al.*, 1988; Van Lookeren Campagne *et al.*, 1991).

The synthesis of most proteins are decreased in the hours after starvation, but the synthesis of several proteins is transiently induced. Abrupt changes in environment and starvation caused an immediate unloading of mRNA from polysomes and an increase in monomeric ribosomes (Margolskee and Lodish, 1980).

The cell cycle cessation is the major event during starvation, however mitochondrial DNA synthesis continues (Shaulsky and Loomis, 1995). As cell proliferation is finely regulated by extracellular signals such as growth factors, there are some checkpoints monitoring the exact progression of cell cycle. It has been shown that a specific checkpoint regulating the transition from growth to development in tumor cell exists in the G1 phase (Sherr, 1996). Although the cell cycle of *Dictyostelium* is regulated by the same components that regulate yeast or other cell cycles (Weeks and Weijer, 1994), it is not known that how it is shut off during starvation. The elucidation of relations between developmental signals and their pathway toward the regulations of genetic program must provide insights into general mechanisms for the initiation of cell differentiation.

2.3. Intracellular signals required for the initiation of development

During the growth phase, *Dictyostelium* cells continuously synthesize and secrete autocrine factors that accumulate in a cell-density-dependent

manner. At appropriate concentrations these factors induce changes in gene expression and prepare cells for the initiation of development. There are two density-sensing mechanisms that function during the early stages of development. One mechanism is the prestarvation response which is mediated by several prestarvation factors (PSFs) and that controls induction of certain very early genes (Rathi and Clarke, 1992). The other mechanism, mediated by conditioned medium factors (CMFs) (Gomer *et al.*, 1991; Iijima *et al.*, 1995), helps the cells to assess their density at a slightly later stage during aggregation. Those diffusible factors secreted during the growth or early development phase work as intercellular communicators that enables starving *Dictyostelium* cells to develop properly.

2.3.1. Prestarvation factors

Prestarvation factors (PSFs) are glycoproteins with a mass of 65-70 kDa and are sensitive to proteases and to heat. PSFs are synthesized during growth and accumulate in the microenvironment according to the density of the cells. Cells can detect the levels of PSFs secreted by growing cells and thus estimate their own density relative to the abundance of external nutrients (Clarke *et al.*, 1988; Maeda and Iijima, 1992; Morita *et al.*, 2004). The prestarvation response occurs during increases in PSFs levels and decreases in nutrients. PSFs regulates the expression of genes involved in sensing cAMP for the oncoming process of aggregation. For example, the expression of *pdsA*, which encodes the secreted cyclic nucleotide phosphodiesterase (ePDE) is induced by PSFs (Lacombe *et al.*, 1986). The ePDE is responsible for resetting the gradient sensing machinery and allowing cells to respond to additional chemotactic signals by degrading extracellular cAMP. The

expression of genes involved in aggregation such as members of the discoidin I gene family, cell adhesion molecule gp24, and lysosomal protein α-mannosidase gene (*manA)* is also induced by PSFs (Clarke *et al.*, 1987; Schatzle *et al.*, 1992). The expression of *carA* encoding the major cAMP receptor during early development is also regulated in this manner (Louis *et al.*, 1993; Rathi *et al.*, 1991; Sun and Devreotes, 1991). The gene inductive effect of PSF is inhibited by the presence of bacteria or the presence of nutrient source. Although the receptor of PSFs has not been identified, the response has been shown to partly depend on signaling to G proteins. In the G protein-dependent pathway, the folate released from bacteria downregulates PSF signaling (Mahadeo and Parent, 2006). The synthesis of PSF declines as development proceeds. PSFs do not promote further development in the absence of starvation.

2.3.2. Conditioned medium factors

Other secreted glycoproteins, conditioned medium factors (CMFs), may play a role in the growth to development transition. When food is depleted, cells stop growing and activate the starvation responses by secreting CMFs. Secreted CMFs are needed to activate cAMP signaling and to initiate aggregation. (Mann and Firtel, 1989; Gomer *et al.*, 1991; Yuen *et al.*, 1995). CMFs are glycoproteins with a molecular mass of 80 kDa (Gomer *et al.*, 1991). Growth-phase cells are able to synthesize CMFs but do not secrete it to growth medium. Upon exhaustion of nutrients, however, starved cells simultaneously secret and sense CMFs to monitor the local cell density during early differentiation. Just before the aggregation stage, CMFs continuously induce early developmental genes such as *discoidin I* and

mediate the expression of a number of early developmentally regulated genes for cAMP pulses and chemotaxis. According to Yuen *et al*. (1995), although cAMP receptors are present in the absence of CMF, the responses to cAMP pulses such as the activation of Ca^{2+} influx, adenylyl cyclase, and guanyly cyclase are strongly inhibited in cells lacking CMFs. The activations are restored by exposure to exogenous recombinant CMFs. The activation of phospholipase C (PLC) by cAMP pulses is not affected by the presence of CMFs (Yuen *et al*., 1995). The interaction of the cAMP receptor with G proteins is also not affected by CMF. However, the activation of adenylyl cyclase by GTPγS requires cell to have been exposed to CMFs, indicating that CMFs controls cAMP signal transduction. CMFs regulates cell aggregation by mediating cAMP signaling at a step after cAMP induces Gα2 to exchange GDP for GTP, but before Gα2 GTP activates adenylyl cyclase.

2.4. The early events induced by starvation

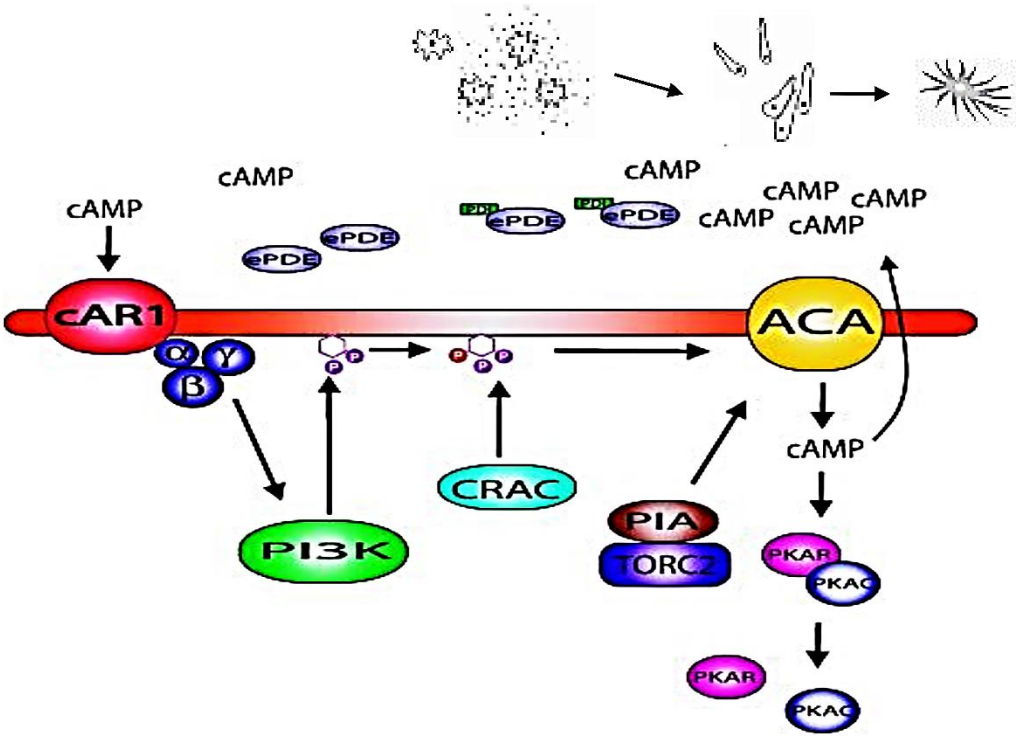
Starvation is an environmental element essential to triggering cell differentiation and a series of morphogenesis in *Dictyostelium*, but it is not enough for the initiation of development. The signals guided by starvation must be integrated into specific events coupled with the transition from growth to development in *Dictyostelium*. During aggregation the cells begin to differentiate into several types with different signaling and chemotactic properties.

2.4.1. The cAMP signaling pathway

Dictyostelium morphogenesis starts with the chemotactic aggregation of starving individual cells. The regulation of this crucial stage of

development revolves around the production, secretion, and inactivation of cAMP. The activation of key components of the cAMP signaling system, such as the major cAMP receptor (cAR1) and the aggregation-stage adenylyl cyclase A (ACA), is one of the earliest responses to starvation in *Dictyostelium*. The cAMP signal relay system employed during aggregation is essential for the development of *Dictyostelium* (Scheme 5). When nutrients are depleted, the cells stop growing and activate starvation responses by secreting a glycoprotein called conditioned medium factor (CMF). Secreted CMF activates cAMP signaling (Mann and Firtel, 1989; Gomer *et al.*, 1991; Yuen *et al.*, 1995). Certain starved cells secrete cAMP, which stimulates neighboring cells to migrate toward cAMP in a head to tail fashion until an aggregate is formed and propagates the cAMP signal to neighboring cells. cAMP gradient is established by the exquisite regulation of the synthesis and degradation of cAMP. cAR1 recognizes secreted cAMP and induces the production of additional cAMP by activating ACA (Van Haastert, 1995). The adaptation process undergoes after stimulation. The transient refractory period the cAMP signaling system is responsible for the outward propagation of cAMP waves because cells which have just relayed the signal are refractory to further stimulation by cAMP. Binding of cAMP to the receptor causes chemotactic movement in the direction of higher cAMP concentration. In addition, *Dictyostelium* cells degrade the extracellular cAMP by using an intricate removal system, ePDE, to prevent the loss of directional information and gene expression resulting from saturation of the receptors.

The cAR1 receptor is essential to aggregation A central element in the chemotactic mechanism is the cAMP receptor, cAR1. The cAMP



Scheme 5. The cAMP signaling pathway during aggregation in *Dictyostelium.* cAMP binding to cAR1 leads to the activation of PI3K and the recruitment of the cytoplasmic, PH-domain containing protein, CRAC. CRAC and other cytoplasmic proteins act in concert to allow the G protein-dependent stimulation of ACA (Mahadeo and Parent, 2006). cAR1, cAMP receptor 1; PI3K, phosphatidylinositol 3-kinase; CRAC, cytosolic regulator of adenylyl cyclase; PIA, pianissimo; TORC2, target of rapamycin complex 2; ACA, adenylyl cyclase; PKAC, protein kinase catalytic subunit; PKAR, protein kinase regulatory subunit; ePDE, extracellular phosphodiesterase.

receptor is a seven trans-membrane domain glycoprotein. It is related to receptors in animals, plants and other simple eukaryotes and is coupled to trimeric GTP-binding proteins (Parent and Devreotes, 1996). There are four receptors for extracellular cAMP (cAR1−4) that are sequentially expressed during *Dictyostelium* development. These receptors display different cell type specificities and different affinities for cAMP. cAR1 and cAR3 have high affinity for cAMP, whereas cAR2 and cAR4 have low affinities for cAMP (Louis *et al.*, 1994; Klein *et al.*, 1988; Saxe *et al.*, 1991; Johnson *et al.*, 1991, 1993). The high affinity cAMP receptors cAR1 is the first to be expressed and the main receptor required for aggregation (Klein *et al.*, 1988; Sun *et al.*, 1990; Sun and Devreotes, 1991). The other high affinity cAMP receptor, cAR3 is partially redundant and can mediate most cAR1-dependent signaling (Insall *et al.*, 1994). Deletion of either gene result in cells that cannot aggregate. The low affinity cAMP receptors cAR2 and cAR4 control events during later times in development (Saxe *et al.*, 1993).

The heterotrimeric G proteins coupled to the cAMP receptors are composed of α, β, and γ subunits. In *Dictyostelium*, G protein complexes may contain 1 of 11 α subunits coupled to a single βγ subunit (Lilly *et al.*, 1993; Wu *et al.*, 1995; Zhang *et al.*, 2001). During aggregation, only Gα2 seems to be coupled to cAR1 and cAR3 to mediate all the cAMP-dependent responses (Kumagai *et al.*, 1989; Sun and Devreotes, 1991). cAMP binding to cAR1/cAR3 induces the exchange of GDP for GTP in the Gα2 subunit and the dissociation of Gα2 from Gβγ. Upon cAMP binding to the receptor, the signal is transduced into the cell through heterotrimeric G-protein-dependent and also independent pathways. Activation of adenylyl cyclase, guanylyl cyclase, and PLC, and modulation of the actin and myosin cytoskeletons are

G protein-dependent effects of cAMP, whereas receptor phosphorylation, Ca^{2+} mobilization, and ERK activation are events that are activated independently of G proteins (Kesbeke *et al.*, 1988; Milne and Coukell, 1991; Milne and Devreotes, 1993; Maeda *et al.*, 1996; Milne *et al.*, 1997; Segall *et al.*, 1995). One of the consequences of cAMP binding to the receptor is cAMP synthesis and secretion. The amount of cAMP made by adenylyl cyclase is proportional to the level of the extracellular stimulus, and the amount of extracellular cAMP depends on how much intracellular cAMP was made (Dinauer *et al.*, 1980).

After cAMP binding on the receptor, there is an adaptive process; a constant level of cAMP causes one burst of synthesis and secretion and then halt. There is no loss of cAR1 from the cell surface. As with many sensory processes, the receptors quickly adapt within ~1−2 min. The adapted receptors, which still bind cAMP, no longer activate intracellular signaling. Within minutes, the extracellular cAMP is hydrolyzed by an extracellular phosphodiesterase (ePDE), allowing the receptors to deadapt and prepare to respond to the next cAMP pulse. In *Dictyostelium*, the alternation between activation and adaptation is essential for relaying the directional cAMP signal necessary for chemotaxis (Devreotes and Zigmond, 1988).

The ACA is highly regulated during aggregation Three distinct adenylyl cyclases are expressed throughout the *Dictyostelium* developmental program; ACA, ACB, and ACG. ACA shares homology with the mammalian G protein-coupled adenylyl cyclases containing two sets of six transmembrane segments each followed by a highly conserved catalytic domain. ACA is expressed during early development and provides the

majority of the cAMP that controls gene induction and aggregation. Remarkably, in addition to the catalytic activity of ACA, the localization of enzyme activity may be important for signal relay. Live cell imaging has shown that ACA is enriched at the rear of chemotaxing cells, suggesting that the mechanism of signal relay may involve restriction of activity to the posterior of cells (Kribel *et al.*, 2003). ACA activity is tightly regulated by the action of the Gα2βγ heterotrimeric complex and two cytoplasmic proteins, cytosolic regulator of adenylyl cyclase (CRAC) and pianissimo. CRAC is a 78 kDa protein containing a N-terminal Pleckstrin Homology (PH) domain that mediates recruitment to the plasma membrane of the leading edge of chemotactic cells on chemoattractant stimulation (Insall *et al.*, 1994; Lilly and Devreotes, 1994, 1995) and it acts as an adaptor between the βγ− complex and ACA (Parent et al., 1998). Pianissimo is also essential for the activation of ACA. The *Pia* gene encodes the *Dictyostelium* homolog of Rictor, a key member of the TORC2 (target of rapamycin complex 2) in mammalian and *Drosophila* (Chen *et al.*, 1997; Sarbassov *et al.*, 2004). It may be required to mediate the binding of CRAC, or act after CRAC has bound to the membrane (Parent *et al.*, 1998). Cells lacking CRAC or Pianissimo are unable to activate adenylyl cyclase and do not aggregate when starved.

ACB is required at the culmination stage, providing much of the cAMP to drive terminal differentiation. Adenylyl cyclase G (ACG) is a unique adenylyl cyclase as it resembles membrane bound guanylyl cyclase in topology (Pitt *et al.*, 1992). ACG contain a large extracellular loop connected to a transmembrane region followed by a single cytoplasmic catalytic loop. The regulation of ACG occurs through an intramolecular switch in the

extracellular loop in response to elevated osmolality in the fruiting body (Saran and Schaap, 2004). ACG is only expressed at the terminal stages of spore formation and its activity helps to maintain spores in a dormant state (Cotter *et al.*, 1999; van Es *et al.*, 1996).

The PKA is essential for gene induction and regulates events in the cytoplasm cAMP-dependent protein kinase A (PKA) plays a critical role during early stage of development and at all later stages. Protein kinase A is a central mediator of development that regulates the levels of expression of genes that respond to cAMP signaling. (Harwood *et al.*, 1992). The PKA of *Dictyostelium* is a dimmer consisting of one regulatory (PKA-R) and one catalytic subunit (PKA-C), rather than the tetramer of higher organisms (de Gunzburg *et al.*, 1986; Anjard *et al.*, 1993). The mRNA for the regulatory subunit of the PKA is present at low levels during growth and the levels increase 10−20 fold during the first 3 hours of development. It continues during the entire course of development (Mutzel *et al.*, 1987). The expression of *pkaC* encoding catalytic subunit of PKA is activated at the onset of development following starvation. Increased *pkaC* expression parallels the activity of PKA and triggers the expression of ACA and cAR1 (Mutzel *et al.*, 1987; Schulkes and Schaap, 1995; Wu *et al.*, 1995; Mann *et al.*, 1997). Overexpression of PKA-C (Anjard *et al.*, 1992; Mann *et al.*, 1992) or the disruption of PKA-R, releasing the catalytic subunit from inhibition, induces rapid development (Abe and Yanagisawa, 1983; Simon *et al.*, 1992). While the disruption of PKA-C leads to developmental failure, aggregateless phenotype, without affecting growth (Mann and Firtel, 1991). Although the PKA activity is present in growing cells, it is not crucial for growth since

PKA-C-null mutants are viable. Thus, the fundamental role of PKA is controlling the earlier events to initiate development.

PKA mediates changes in gene expression responding to cAMP signaling. Several of the genes involved in chemotaxis such as *acaA* (adenylyl cyclase), *pdiA* (the phosphodiesterase inhibitor), and *carA* (the major cAMP receptor in early development), are not transcribed at all in the absence of the PKA catalytic subunit (Mann *et al.*, 1997; Wu *et al.*, 1995). Recent evidence suggests that the role of PKA is achieved by a series of sensor histidine kinases that integrate with the cAMP signaling events (Loomis, 1998; Thomason *et al.*, 1998).

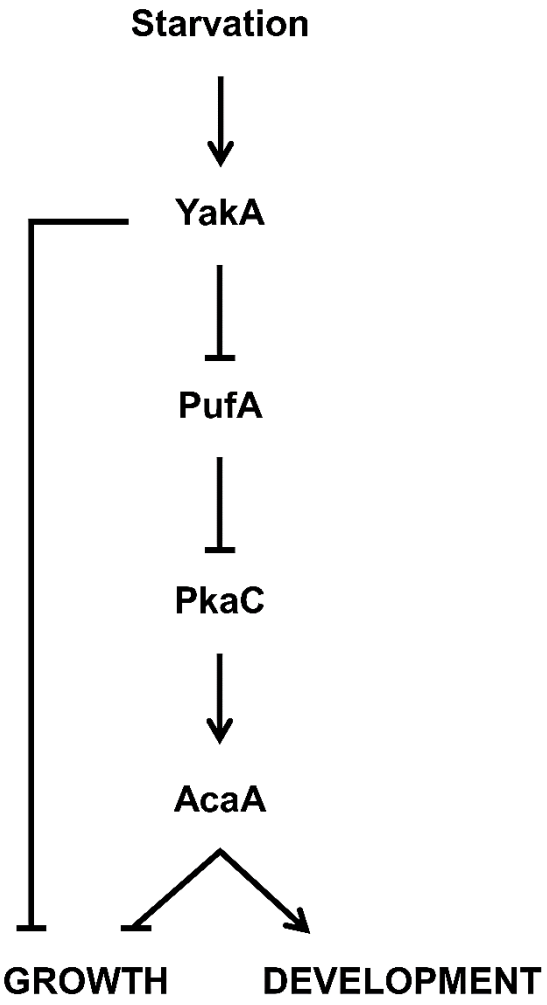
2.4.2. The YakA signaling pathway

The YakA signaling pathway comprises YakA, PufA, and PKA and is activated early when cells are starved. YakA is a member of the dual-specificity tyrosine-related kinase (DYRK) family of serine/threonine kinases and a homologue of yeast Yak1p growth-regulating protein kinase (Souza *et al.*, 1998). The expression of *yakA* is required for turning off growth-phase genes and for induction of differentiation-associated genes. During growth, *yakA* mRNA accumulates and reaches a maximum level at the time of starvation. YakA induces developmental processes such as growth inhibition, downregulates the expression of genes encoding vegetative functions, and upregulates the expression of PKA-C, ACA, and cAR1 (Souza *et al.*, 1998; 1999). *yakA* null cells divide and multiply more rapidly compared to parental cells, reducing their size. PKA-cat mRNA appears normal in *yakA* null cells, but the enzyme activity of PKA does not exhibit the characteristic increase after 5 h of starvation. PKA-dependent

gens are also not expressed in *yakA* null cells. Importantly, *yakA* null cells cannot turn off vegetative genes. The yeast Yak1p is capable of mediating transition from growth to development in *Dictyostelium*, and vice versa, indicating that YakA shares many functions with the yeast Yak1p (Souza *et al.*, 1998).

Other component relating to YakA signaling is PufA. PufA is identified from a mutant which can reverse the aggregateless phenotype of *yakA* null cells to normal. PufA is a member of the Puf (pumilio/FBP) family of proteins, which functions in the translational control of key regulators for anterior-posterior patterning in *Caenorhabditis elegans* and *Drosophila melanogaster* (Forbes and Lehmann, 1998; Wharton *et al.*, 1998; Zamore *et al.*, 1999; Zhang *et al.*, 1997). PufA proteins are sequence-specific RNA binding proteins that bind the 3′ end of mRNA encoding developmentally key regulators. The pumilio protein of *Drosophila* binds to the 3′ end of the hunchback protein and, together with the nanos protein, inhibits the translations of the hunchback protein in the posterior region of oocytes. The RNA sequence (Nanos response elements, NREs) to which pumilio binds have been defined. PKA is likely candidates for regulation by PufA, because *Dictyostelium* PKA mRNA has sequence related to the NRE control elements of the *Drosophila* hunchback protein (Souza *et al.*, 1999).

The YakA signaling regulates the initiation of development by modulating the expression of *yakA* and *pufA*. *pufA* mRNA is present during growth and disappeared by 8 h of starvation. In *yakA* null cells, *pufA* mRNA levels are retained high even after2 h of starvation. Further, the inactivation of *pufA* shows decreased *pufA* expression and allows *yakA* null cells to differentiate, thus indicating that YakA is required for the loss of *pufA* mRNA



Scheme 6. The YakA signaling pathway during aggregation in *Dictyostelium*. Environmental stress signal stimulates the transcriptional expression of YakA. Expressed YakA positively regulates PKA by inhibiting PufA which is proposed to be a direct inhibitor of translation and inhibits the expression of growth-specific genes for the initiation of development of *Dictyostelium* (Taminato *et al*., 2002).

at the onset of development. That is, YakA represses the transcription of *pufA* and thus allows the translation of PKA mRNA to induce development after starvation (Scheme 6). However, genetic perturbations of the signaling events in YakA signaling have not be identified.

3. Aims of this study

GSH is the most prevalent intracellular non-protein thiol compound and performs diverse cellular functions. GSH is essential for survival and differentiation depend on at least some of the multifunctional properties of it. Recently, the critical roles of GSH are reported during growth and development. During growth, the null-mutant of *gcsA* encoding γ-glutamylcysteine synthetase (*gcsA*$^{-}$) shows growth inhibition by methylglyoxal accumulation (Choi *et al.*, 2008) in *Dictyostelium discoideum*. During *Dictyostelium* development, the developmental status is determined by the concentration of intracellular GSH (Kim *et al.*, 2005; Choi *et al.*, 2006). And the expression of *gcsA* is regulated during development, especially it increases during aggregation, indicating that intracellular GSH has role during development of *Dictyostelium*. The evidences for the importance of GSH in early embryonic development are also found in animals and plants. Although GSH has been reported to have a vital role in regulating development, the understanding on the precise action mechanism of GSH is unknown. The elucidation of in what state of development GSH presents a key role and how GSH regulate development will explain fundamental principles of development. The properties of signaling systems which are required for the induction of development in *Dictyostelium* is

remarkably conserved in other higher eukaryotic cells. Thus, the results shown in this study will provide further understanding of general mechanisms of development, in particular the transition from growth to development, not only in *Dictyostelium*, but also in higher organisms.

II. MATERIALS AND METHODS

1. Strains and culture conditions

1.1. *Dictyostelium* strains and culture conditions

1.1.1. *Dictyostelium* strains

Dictyostelium discoideum KAx3 strain was used as a wild-type strain. All mutant strains used in this work were derived from KAx3 cells. γ-Glutamylcysteine synthetase disruption strain (*gcsA*$^{-}$ cells) (Kim *et al.*, 2005), YakA disruption strain (*yakA*$^{-}$ cells), cAMP receptor (cAR1)-expressing strains (cAR1^{OE}/KAx3 and cAR1^{OE}/*gcsA*$^{-}$ cells) and YakA-expressing strains (YakA^{OE}/KAx3 and YakA^{OE}/*gcsA*$^{-}$ cells) were used in this study. The *Dictyostelium* strains used in this study were summarized in Table 1.

1.1.2. Culture condition

Wild-type strain KAx3 and KAx3 mutant cells were grown axenically with shaking at 22 °C in HL5-liquid medium (1.4% thiotone E peptone, 1.4% glucose, 0.7% yeast extract, 3.5 mM $Na_2HPO_4 \cdot 7H_2O$, 4.6 mM KH_2PO4, pH 6.5) containing 100 μg/ml of streptomycin (Duchefa) and 100 units/ml of penicillin (Duchefa) (Cocucci and Sussman, 1970; Soll *et al.*, 1976). To maintain cAR1^{OE}/KAx3, cAR1^{OE}/*gcsA*$^{-}$, YakA^{OE}/KAx3, and YakA^{OE}/*gcsA*$^{-}$ cells, 20 μg/ml of G418 (Duchefa) was supplemented in HL5 medium and to maintain *gcsA*$^{-}$ and *yakA*$^{-}$ cells, 10 μg/ml of blasticidin (ICN) was supplemented in culture medium. For long-term storage of the cells, cell stocks were prepared with 5% DMSO (Sigma-Aldrich)-HL5 and stored at –70 °C. For routinely renewal of cell strain, a frozen stock cell was thawed and suspended in HL5 medium on culture dish plate and used for

experiments by monthly intervals. Every cell culture was harvested during exponential growth.

1.2. Bacterial strains and culture conditions

1.2.1. *Escherichia coli* strains for gene cloning

E. coli DH5α was used for DNA manipulation. DH5α strains were grown at 37 °C on Luria-Bertani (LB, 1% tryptone, 0.5% yeast extract, 1% NaCl) medium with 1.5% agar, where required with the following antibiotics at final concentrations: 50 μg/ml ampicillin (Sigma-Aldrich) and chemicals at final concentrations: 20 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal purchased from Duchefa).

1.2.2. *Klebsiella pneumoniae* strain as a food source of *Dictyostelium*

K. pneumoniae were grown on SM broth (1% glucose, 1% bacto peptone, 1% yeast extract, 4 mM $MgSO_4 \cdot 7H_2O$, 14 mM KH_2PO_4, 5.7 mM K_2HPO_4, prepared as described by Sussman, 1987) with 2% agar. Plates were incubated overnight at 37 °C and stored at 4 °C after sufficient colony growth. Loopful of bacteria harvested from these plates and inoculated for the two-membered *Dictyostelium* cultures. The bacterial strains used in this study were summarized in Table 1.

2. Depletion of GSH

To deplete completely intracellular GSH in *gcsA*$^{-}$ cells, cells were grown exponentially in HL5 media with 1 mM GSH (Duchefa) and were then reinoculated at a density of 2×10^5 cells/ml in media containing 0.5 mM

Table 1. Bacterial and *Dictyostelium discoideum* strains used in this study

Strains	Genotypes	References or sources
Bacterial strains		
E. coli DH5α	F-*ΔlacU169(ϕ80lacZΔM15)endA1 rec1hsdR17 deoR supE44 thi-1 λ-gyrA96 relA1*	Hanahan, 1983
K. pneumoniae		Microbial resources center, 1997
***Dictyostelium discoideum* strains**		
KAx3	Axenic wild-type strain	Firtel, 1997
gcsA^{-}	KAx3:[GCS–Bsr], bs^{r} *gcsA*-disrupted KAx3	Kim, 2005
yakA^{-}	KAx3:[YakA–Bsr], bs^{r} *yakA*-disrupted KAx3	Devreotes, 2001
cAR1^{OE}/KAx3	KAx3:[EXP4(+)–cAR1], neo^{r} cAR1-expressing KAx3	This study
cAR1^{OE}/*gcsA*$^{-}$	*gcsA*$^{-}$:[Exp4(+)–cAR1], neo^{r} cAR1-expressing *gcsA*$^{-}$	This study
YakA^{OE}/KAx3	KAx3:[pTX FLAG–YakA], neo^{r} YakA-expressing KAx3	This study
YakA^{OE}/*gcsA*$^{-}$	*gcsA*$^{-}$:[pTX FLAG–YakA], neo^{r} YakA-expressing *gcsA*$^{-}$	This study

GSH. The exponentially growing cells were inoculated in the same manner in a medium containing 0.2 mM GSH. *gcsA*$^{-}$ cells cultured with 0.2 mM GSH were transferred to fresh HL5 media without GSH and were incubated for 24 h. The experimental scheme is shown in diagram (Fig. 1).

3. Development of *Dictyostelium discoideum*

3.1. Development on non-nutrient agar plates

Development of *Dictyostelium* was induced by removing nutrients. Exponentially growing cells at a density of 2–5 × 10^6 cells/ml were washed twice with non-nutrient KK2 buffer (16.5 mM KH_2PO_4, 3.8 mM K_2HPO_4, pH6.2) and the buffer was removed by centrifugation at 500 g for 5 min. And then the cells were suspended with KK2 buffer and plated at a density of 2 × 10^6 cells/cm^2 on a nitrocellulose filter or on 1.5% KK2 agar plates. Cells plated on agar plates were incubated at 22 °C for desired time. For the development of *gcsA*$^{-}$ cells, exogenous 1 mM GSH was supplemented to KK2 buffer before subjecting on plates.

3.2. Development in non-nutrient buffer

To induce development in suspension, cells were washed with KK2 buffer twice and the buffer was removed by centrifugation at 500 g for 5 min. And then washed cells were suspended in non-nutrient KK2 buffer at a density of 1 × 10^7 cells/ml and shaken at 150 rpm at 22 °C. For the development of GSH-depleted *gcsA*$^{-}$ cells, 1 mM GSH (Duchefa) was added to development buffer. After 2 h of development, cAMP stimulation was performed by adding of 30 nM cAMP (Sigma-Aldrich) every 6 min. 30 nM

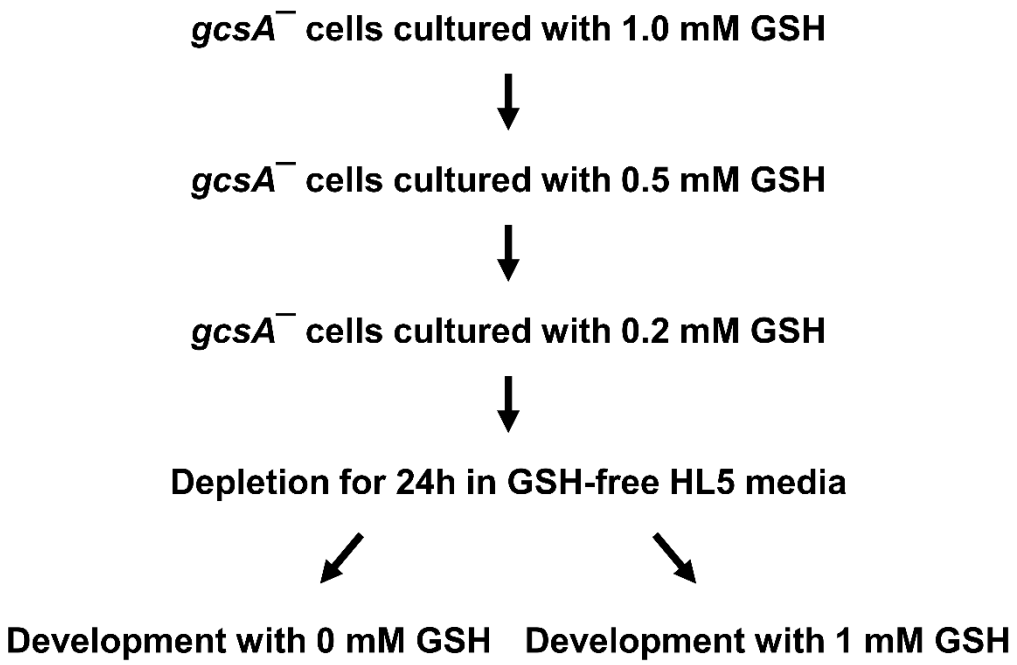


Fig. 1. Experimental scheme for the complete depletion of intracellular GSH and for the development of GSH-depleted *gcsA*¯ cells. Intracellular GSH was depleted from the cells gradually and GSH-depleted *gcsA*¯ cells were subjected on non-nutrient KK2 agar or in non-nutrient KK2 buffer.

cAMP was supplemented for 4 h and after then 300 μM cAMP was added every 2 h for 4 h (Galardi-Castilla *et al.*, 2008). The experimental scheme for the exogenously added cAMP pulses is presented in diagram (Fig. 2).

To examine the effect of other thiol compounds or antioxidant on the development of *gcsA*$^{-}$ cells, 1 mM oxidized glutathione (GSSG purchased from Sigma-Aldrich), 1 mM γ-glutamyl cysteine (γ-GC purchased from Sigma-Aldrich), 1 mM dithiothreitol (DTT purchased from Duchefa), 1 mM *N*-acetylcysteine (NAC purchased from Sigma-Aldrich), and 1 mM ascorbic acid (Sigma-Aldrich) were supplemented to non-nutrient KK2 buffer.

4. Transformation of *Dictyostelium discoideum*

The transformation of *Dictyostelium* cells was performed according to the protocol (Schlatterer *et al.*, 1992) with some modification (Pang *et al.*, 1999). *Dictyostelium* cells were grown axenically in suspension culture to a density of 2–3 × 10^6 cells/ml. Cells were washed twice with an equal volume of ice-cold H-50 buffer (20 mM HEPES, 50 mM KCl, 10 mM NaCl, 1 mM $MgSO_4$, 5 mM $NaHCO_3$, 1 mM NaH_2PO_4, pH 7.0, autoclaved and stored cold or frozen). The washing buffer was removed by centrifugation at 500 × g for 5 min at 4 °C. After washing, the cells were resuspended in H-50 buffer at a density of 5 × 10^6 cells/ml. For electroporation, 1–10 μg of plasmid DNA was added to 100 μl of suspended cells in H-50 buffer and the cell-DNA mixture was transferred to a pre-chilled electroporation cuvette (0.1 cm electrode gap, Bio-Rad). The cells were sparked with electricity at 0.85 kV / 25 μF twice with about 5 sec between pulses. After electroporation, the cells were incubated in the cuvette on ice for 5 min and suspend into a cell culture

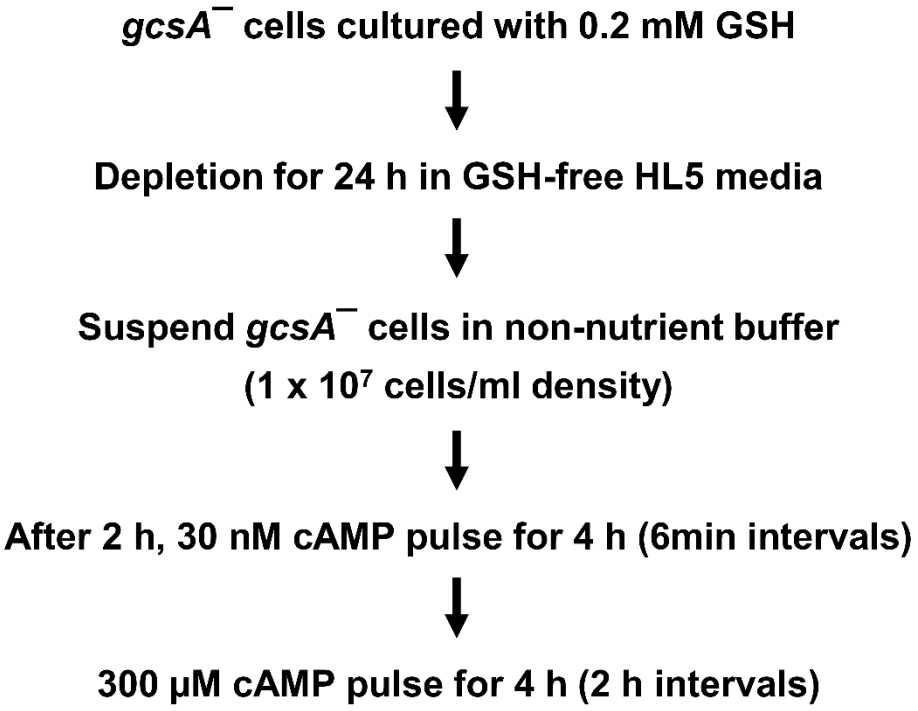


Fig. 2. Experimental scheme for suspension development of GSH-depleted *gcsA*$^{-}$ cells with cAMP pulses. Exogenous cAMP was supplemented as the same condition of internal cAMP secretion at 6 m intervals and at nanomolar levels.

dish with 10 ml of HL5 medium. After 18–24 h incubation at 22 °C, the medium was replaced by the selection medium containing appropriate antibiotics (20 μg/ml of G418 purchased from Duchefa or 10 μg/ml blasticidin S purchased from ICN).

5. Genetic manipulation methods

General techniques for isolation and manipulation of DNA in *E. coli* were as previously described (Sambrook and Gething, 1989). pGEM-T easy vector (Promega) was used for cloning of PCR product. Integrating expression vector Exp4(+) (Dynes *et al.*, 1994) and extrachromosomal expression vector pTX-FLAG (Levi *et al.*, 2000) were used for introducing appropriate genes into *Dictyostelium*. The constructs and plasmids used in this study were summarized in Table 2.

5.1. Isolation and subcloning of *carA* from *Dictyostelium discoideum*

Full-length of *carA* (1.3 kb) was amplified by polymerase chain reaction (PCR) using genomic (g)DNA as the template. The PCR-primer sequences were as follows: forward 5′-GGATCCATGGGTCTTTTAGATGGAAATCC A-3′ and reverse 5′- CTCGAGATCAATTATTTCCTTGACCATTT-3′. The amplified product was cloned into pGEM-Teasy cloning vector (Promega), yielding pGEM–Teasy–cAR1. The construct was digested with *Bam*HI and *Xho*I and a construct for constitutive expression of cAR1 was generated by cloning the full-length gDNA *carA* amplicon into the Exp4(+) vector containing a constitutively active *Act15* promoter. The constructs were

Table 2. Plasmids and constructs used in this study

Plasmids	Descriptions	References or sources
pGEM-Teasy	PCR cloning vector	Promega
Exp4(+)	Expression vector for *Dictyostelium*	Firtel, 1997
pTX–FLAG	FLAG-tagged protein expression vector	Egelhoff, 2000
pGEM–Teasy–cAR1	pGEM–T easy vector containing *carA* ORF	This study
Exp4(+)–cAR1	Exp4(+) vector fused with *carA* ORF in frame	This study
pGEM–Teasy–YakAF1	pGEM–T easy vector containing fragmented *yakA* ORF (1-2621)	This study
pGEM–Teasy–YakAF2	pGEM–T easy vector containing fragmented *yakA* ORF (2622-4377)	This study
pGEM–Teasy–YakA	pGEM–T easy vector containing full-length of *yakA* ORF	This study
pTX-FLAG–YakA	pTX–FLAG vector fused with *yakA* ORF in frame	This study

introduced into KAx3 or *gcsA*$^{-}$ cells using electroporation (Pang *et al.*, 1999), and transformants were selected and maintained in medium containing 10 μg/ml of G418 (Duchefa).

5.2. Isolation and subcloning of *yakA* from *Dictyostelium discoideum*

Full-length of *yakA* (4.3 kb) amplified into two fragments by polymerase chain reaction (PCR) using complementary (c)DNA as the template: first fragment (YakAF1) with *Sac*I and *Bam*HI restriction enzyme site, second fragment (YakAF2) with *Bam*HI and *Xho*I restriction enzyme site. These two fragments were ligated and cloned into pGEM-Teasy cloning vector (Promega), yielding pGEM–Teasy–YakA. The primer sequences of *yakA* fragment 1 (1 to 2621) for PCR were as follows: forward 5′-CAATAGAGCTCATGGGCAGTACTACACAAATGAGC-3′ and reverse 5′-GTGGATCCATTCCCTCTGAACTTG-3′. The primer sequences of *yakA* fragment 2 (2622 to 4377) were as follows: forward 5′-CAAGTTCAGAGG GAATGGATCCAC-3′ and reverse: 5′-GTATATATTTTCTCGAGTTATGTC TCTCTATATGAACCAATAACAACC-3′. The construct was digested with *Sac*I and *Xho*I and a construct for constitutive expression of cAR1 was generated by cloning the full-length cDNA *yakA* amplicon into the pTX-FLAG vector containing a constitutively active *Act15* promoter. The constructs were introduced into KAx3 or *gcsA*$^{-}$ cells using electroporation (Pang *et al.*, 1999), and transformants were selected and maintained in medium containing 10 μg/ml of G418 (Duchefa).

5.3. Polymerase chain reaction (PCR)

DNA fragment amplification was performed according to the method recommended by Taq polymerase manufacturer (Promega, Madison, WI) with slight modification. For the reaction, 25 pmol of degenerate oligonucleotide primers, 100 ng of cDNA or gDNA and 0.25 units of Taq polymerase were combined in a final volume of 25 μl with reaction buffer (50mM KCl, 1.2 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.4, 0.01% gelatin) containing 50 μM of each dNTP. The mixture was subjected to 30 cycles of 30 sec denaturation at 94 °C, 30 sec annealing at 55 °C and 1 min extension at 72 °C.

5.4. Real-time reverse transcriptase-polymerase chain reaction (Real-time RT-PCR)

Each RNA sample (50 ng/μl) was reverse-transcribed into cDNA using superscript III Reverse Transcriptase Kit (Promega). Real-time PCR was performed in a 20-μl volume in the well of 96-well reaction plates (Bioplastics). Each PCR assay was performed using SYBR Premix Ex Taq (TaKaRa), and *rnlA* served as an endogenous control. Fluorescence was detected using an Applied Biosystems 7500 real-time PCR system. The reactions for each gene at each time point were performed in triplicate, and cycle threshold values generated from the reactions were averaged. The cycle threshold values of each gene were normalized to the endogenous controls and calibrated to an average expression level for the gene being analyzed (Kim *et al.*, 2011). The primer sequences of *yakA* for real-time RT-PCR were as follows: forward 5′-CACCTTTGATGATGTCACAACCAC-3′ and reverse 5′-ATAGAAGATGCATCACCCATCAATG-3′. The primer

sequences of *rnlA* were as follows: forward 5′-ACTAGGCAGACTATGAG CGCTAAGG-3′ and reverse: 5′-CTGTAGATTGTTGGCTAGAGAAC-3′.

5.5. RNA extraction and Northern blot analysis

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the supplier's recommendations and solubilized in formamide (Sigma-Aldrich). The RNA (20 μg) sample was separated by electrophoresis through a 1% agarose gel containing 0.22 M formaldehyde (Sigma-Aldrich) and then transferred to Hybond-N+ nylon membrane (GE Healthcare). The specific probes were labeled with [α-^{32}P]-dATP (Feinberg and Vogelstein, 1983). The primer sequences of each probes for hybridization were summarized in Table 3. Hybridization was performed using various probes dissolved in Rapid-Hyb buffer (GE Healthcare) according to the manufacturer's instructions. The blots were incubated in Rapid-Hyb buffer without the probe for 1 h and then probe was added for 2 h. The blot was washed twice with SSC buffer (0.1% SDS, 0.3 M NaCl, 30 mM trisodium citrate) for 10 min at 65 °C. The signal was visualized by exposing membrane to X-ray film. All the solutions were treated with diethyl pyrocarbonate (DEPC purchased from Sigma-Aldrich) and autoclaved.

6. Measurement of PKA activity

PKA activity measurements were performed as described previously (Wang and Kuspa, 2002) using the SignaTECT PKA Activity System (Promega). Samples were prepared from cells developed in non-nutrient KK2 buffer with cAMP pulses. Harvested cells by centrifugation at 500 ×

Table 3. List of primer sequences used in Northern blot analysis for the preparation of hybridization probes

Probes	Primer sequences (5′ to 3′)	Position
cprD	F[a]: CAGCCCTCATTGGTACTGAAGAAG R[b]: CGATCCAGTAGTTACCAGATGAGGCC	290 to 1210
carA	F: GAGAACCAGAACCAGAAAGATTTG R: TTTCCTTGACCATTTGTTGAAGTGG	332 to 1172
acaA	F: GGATCCTGCACCTTATTTCAATAG R: CTCGAGATTTGGTTAATGCAGATTGTGGG	2952 to 3499
gpaB	F: ATGGGTATTTGTGCATCATCAATGGAAG R: CAGTTGGAATATAAACTGGTGATGTCATACG	1 to 523
pkaC	F: GAATTCTCAAGGTCACATTAAAATCACTG R: GAATTCGGAGGCTCTTCAACCATTTCTTC	1407 to 1898
pkaR	F: GGATCCATGACAAATAATATATCACATAACC R: CTCGAGTTAAGATTTTTGAGAGGTTAAATTTGG	1 to 984
pufA	F: CACCCTGTAGTTACATTATCATCATCAC R: GGTTGTTGCTGCTGATGACAATGATGACG	1120 to 1620
dscA	F: ATGTCTACCCAAGGTTTAGTTC R: TTATTCCAAAGCGGTAGCAATG	1 to 762
dia2	F: ATGAAACAAATTATTAGATTAATAACTAC R: GTTTGGAATAACTTGATATAATTTTCCAG	1 to 453
gcsA	F: CGATGATGAAAAGAATACAGATC R: TTAACAATAATAATCATCTTTATC	1200 to 1881
rnlA	F: GGCGGAACCCGTAACTGTTGCAAAAG R: CACAATTATACGGAACATTTTACTACC	695 to 1216

[a] F, forward primer; [b] R, reverse primer.

g for 5 min at 4 ℃ were sonicated and supernatant were separated by centrifugation at 12,000 rpm for 15 min at 4 ℃. Cell extracts containing 10 μg of protein were prepared as specified by the manufacturer and were used in reactions with 10 μM cAMP and in the presence or absence of 20 μM of the PKA-specific inhibitor PKI (Mann *et al.*, 1992). PKA activity is defined as the amount of the phosphorylated substrate, kemptide (nmol/min/mg of protein), in the absence of PKI minus the amount of phosphorylated substrate in the presence of PKI.

7. Measurement of glutathione concentration

To determine the concentration of intracellular glutathione, cell extracts were reacted with monobromobimane (mBBr) to form derivates and then analyzed using a modification of method described by Newton and Fahey (1995). Cells developed in non-nutrient KK2 buffer in suspension and were harvested by centrifugation at 500 × g for 5 min at 4 ℃. Prepared cells were extracted with 50% aqueous acetonitrile (Sigma-Aldrich) containing 50 mM Hepes (pH 8.0), 2 mM EDTA, and 2 mM mBBr (Sigma-Aldrich). After incubation at 60℃ for 15 min, the samples were acidified with 5 μl of 5 N methanesulfonic acid (Sigma-Aldrich). Cell debris was removed from the crude extract by centrifugation at 12,000 rpm for 15min, and the resulting supernatant was analyzed using HPLC. Control samples were treated with 5 mM *N*-ethylmaleimide (NEM purchased from Sigma-Aldrich) and incubated for 10 min before derivatization to prevent labeling of thiol group from with mBBr. The concentration of total GSH is determined using 2 mM dithiothretol (DTT), which reduces GSSG to GSH. Samples (10 μl) were

passed through a ZORBAX SB-C18 column. HPLC was performed using a Waters system equipped with a Hewlett-Packard 1050 series fluorescence detector. The mBBr-derived thiol compounds were detected using excitation and emission at 370 and 480 nm, respectively. The mobile phase consisted of buffer A (methanol, HPLC grade from Sigma-Aldrich) and buffer B (0.1% trifluoroacetic acid from Sigma-Aldrich). The proportion of buffer A in the continuous gradients was as follows; 15% at 0–2 min, 25% at 30 min, 100% at 34 min, 15% at 37 min, and 15% at 40 min. If necessary, samples were co-injected with GSH (Duchefa) standards.

III. RESULTS

1. The roles of GSH in development of *Dictyostelium discoideum*

1.1. Complete depletion of GSH in *Dictyostelium*

Previously, it is demonstrated that GSH is essential for the normal development of *Dictyostelium* (Kim *et al.*, 2005). *gcsA*$^{-}$ cells exhibit different developmental morphologies as GSH concentration which is exogenously added to culture media before development. *gcsA*$^{-}$ cells are arrested at mound stage when pre-cultured with 0.2 mM GSH and at culmination step when pre-cultured with more than 0.5 mM GSH. Prespore-specific genes and spore-specific genes are not expressed in *gcsA*$^{-}$ cells. In the present study, to address GSH functions in what developmental stage and how regulates the development of *Dictyostelium*, developmental morphology was observed when intracellular GSH was depleted completely. For the complete depletion of GSH in *gcsA*$^{-}$ cells, cells grown in HL5 media with 1 mM GSH were re-inoculated to media with 0.5 mM GSH, and then to 0.2 mM GSH gradually. Finally, cells cultured with 0.2mM GSH transferred to fresh HL5 media with no GSH and incubated for 24h as mentioned in materials and methods. The depletion of GSH was confirmed by measuring the concentration of intracellular GSH using HPLC connected to a fluorescence detector. The intracellular GSH level was not detected in *gcsA*$^{-}$ cells with no addition of GSH (Fig. 3). It was recovered to around 60% of KAx3 in *gcsA*$^{-}$ cells when cultured with the exogenous addition of 1 mM GSH. Intracellular GSH was removed efficiently and furthermore, an empirical study of the role of GSH in the regulation of development of *Dictyostelium* was possible by producing the GSH-depleted cells.

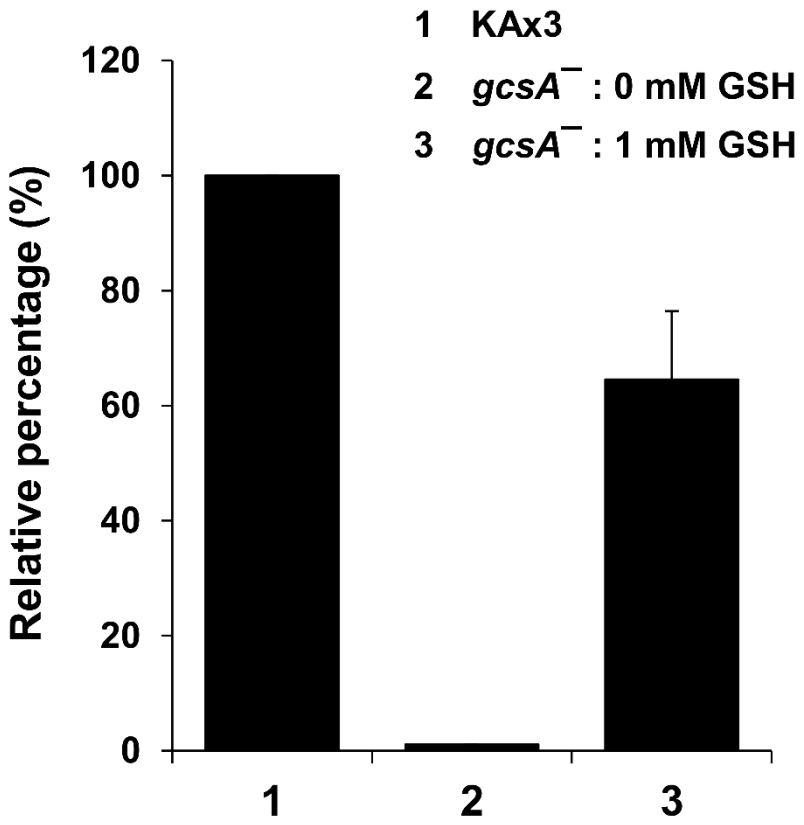


Fig. 3. Complete depletion of intracellular GSH. The concentration of intracellular GSH was measured in exponentially growing KAx3 cells, GSH-depleted *gcsA*¯ cells (*gcsA*¯: 0 mM GSH), and *gcsA*¯ cells which were cultured with 1 mM GSH (*gcsA*¯: 1 mM GSH) using HPLC and fluorescent detector. Intracellular GSH was modified to a mBBr-conjugated form to detect. The concentration of GSH was calculated in relative values compared to that of KAx3 cells. The values represent the mean ± S.E.M. of three independent experiments.

1.2. The roles of GSH in development on agar plates

Developmental morphology of GSH-depleted *gcsA*$^{-}$ cells was observed in the presence of GSH or in the absence of GSH. GSH-depleted *gcsA*$^{-}$ cells did not develop without the addition of GSH when subjected on non-nutrient KK2 agar plates (Fig. 4). However, they formed the final developmental structure, fruiting bodies, with the addition of 1 mM GSH. The efficiency of fruiting body formation and the viability of spore were much lower than those of wild-type KAx3 cells as described by Kim *et al.* (2005). Because the GSH-depleted *gcsA*$^{-}$ cells did not initiate development, the early developmental state, aggregation process, was monitored closely using a phase-contrast microscope. KAx3 cells agglomerated together to form aggregates and formed tipped aggregates at 12 h in response to starvation signal (Fig. 5). *gcsA*$^{-}$ cells did not aggregate without GSH but formed aggregates clearly with distinct stream patterns when 1 mM GSH was exogenously added though the process was slightly delayed compared to KAx3 cells. According to these results, it is clear that GSH regulates development of *Dictyostelium*, particularly the initiation of development.

1.3. The roles of GSH in aggregation processes

There were limits on observing the procedures of formation of aggregates on non-nutrient agar plates. For detailed analysis, cells were induced to develop in suspension, because this is an effective method for observing early developmental processes, especially cell aggregation. KAx3 cells developed and formed aggregates in non-nutrient KK2 buffer (Fig. 6). However, *gcsA*$^{-}$ cells did not form aggregates in the absence of added GSH

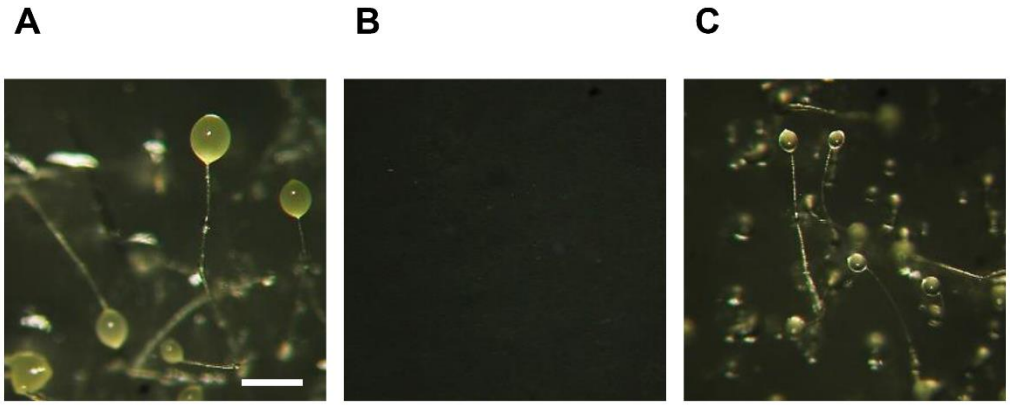


Fig. 4. Developmental morphology of KAx3 and *gcsA*$^{-}$ cells on non-nutrient KK2 agar plates. KAx3 cells and GSH-depleted *gcsA*$^{-}$ cells were allowed to develop on non-nutrient KK2 agar plates with or without the addition of 1 mM GSH under overhead lightening and photographed at 24 h after development. (A) KAx3 cells, (B) *gcsA*$^{-}$ cells without GSH, (C) *gcsA*$^{-}$ cells with 1 mM GSH. The scale bar represents 0.25 mm.

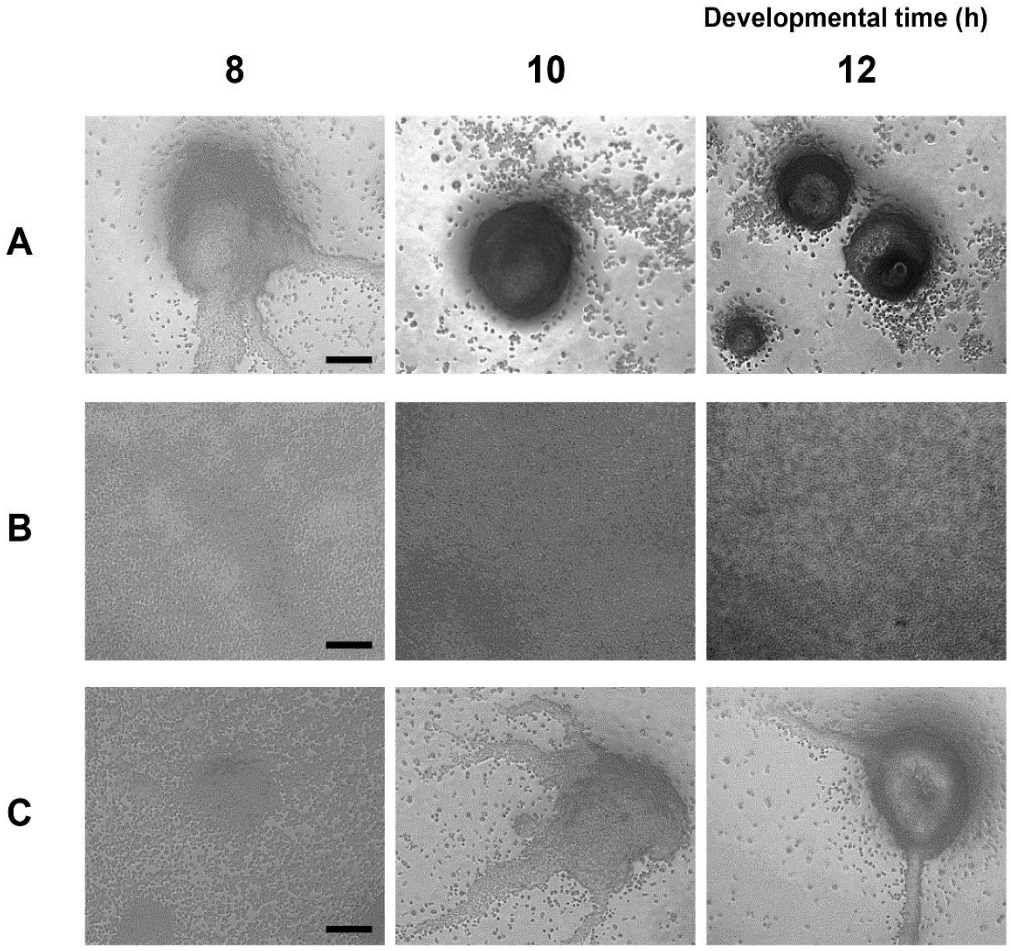


Fig. 5. Developmental morphology of KAx3 and *gcsA*$^{-}$ cells during aggregation on non-nutrient KK2 agar plates. KAx3 and *gcsA*$^{-}$ cells were subjected on KK2 plates with or without the addition of 1 mM GSH under an overhead light. The process of aggregation was observed at the indicated time using a phase-contrast microscope. (A) KAx3 cells, (B*) gcsA*$^{-}$ cells without GSH, (C) *gcsA*$^{-}$ cells with 1 mM GSH. The scale bar represents 0.1 mm.

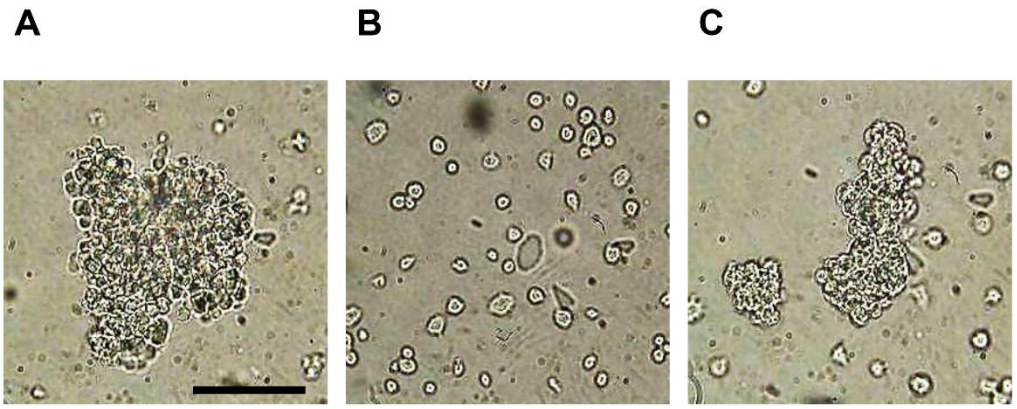


Fig. 6. Developmental morphology of KAx3 and *gcsA*$^{-}$ cells in suspension without cAMP pulses. KAx3 and *gcsA*$^{-}$ cells were allowed to develop in non-nutrient KK2 buffer in the presence of 1 mM GSH or in its absence and photographed at 12 h. (A) KAx3 cells, (B) *gcsA*$^{-}$ cells without GSH, (C) *gcsA*$^{-}$ cells with 1 mM GSH. The scale bar represents 0.05 mm.

and consistently remained as single cells. *gcsA*$^{-}$ cells formed aggregates in the presence of 1 mM GSH, although they were small compared with KAx3 cells. To examine that restoration of the developmental defect of GSH-depleted *gcsA*$^{-}$ cells was due to the supplementation of exogenous GSH, the intracellular GSH level was monitored after treatment of 1 mM GSH in *gcsA*$^{-}$ cells during suspension development. It was approximately 10% of that in KAx3 cells after commencement of starvation (0 h) and increased gradually up to approximately 40% at 4 h after the supplementation of GSH (Fig. 7). The level was not increased further and was sustained. In *gcsA*$^{-}$ cells without the addition of GSH, intracellular GSH was not detected. The relatively low intracellular GSH levels in *gcsA*$^{-}$ cells to the levels in KAx3 cells in the presence of the added 1 mM GSH may explain delayed developmental processes, as shown in Figs. 3, 4, and 5. These results indicate that GSH functions for the developmental initiation in *Dictyostelium*.

1.4. Irreplaceable roles of GSH by other antioxidant molecules

In addition to exogenous GSH, the effect of a precursor of GSH biosynthesis, γ-GC (γ-glutamylcysteine), and oxidized form of glutathione, GSSG, on the developmental morphology of GSH-depleted *gcsA*$^{-}$ cells was examined. *gcsA*$^{-}$ cells aggregates in the presence of both 1 mM γ-GC and GSSG, though the rate of formation of aggregates was significantly slow than that of KAx3 and *gcsA*$^{-}$ cells with the addition of GSH (Fig. 8). These results indicate that not only GSH but also the compounds which could be converted to GSH inside of cells rescue the developmental defects of *gcsA*$^{-}$ cells.

The role of GSH as an important antioxidant via its potent reducing

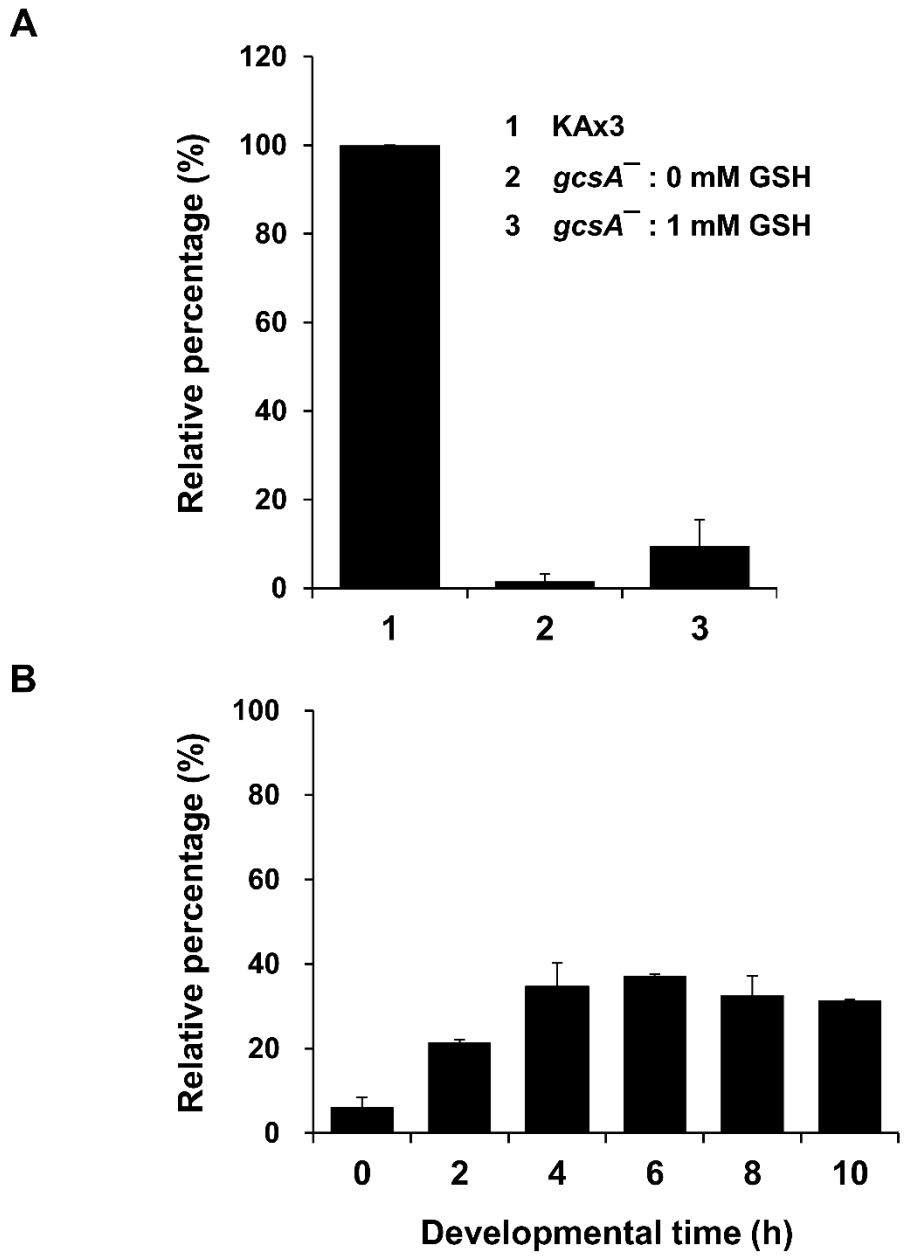


Fig. 7. Intracellular GSH concentration of *gcsA*¯ cells during suspension development. (A) The relative values of the GSH concentration in *gcsA*¯ cells without or with 1 mM GSH compared to that of KAx3 cells at 0 h of development. (B) The relative values of the GSH concentration of *gcsA*¯ cells with 1mM GSH during aggregation compared to that of KAx3 cells. The values represent mean ± S.E.M. of three independent experiments.

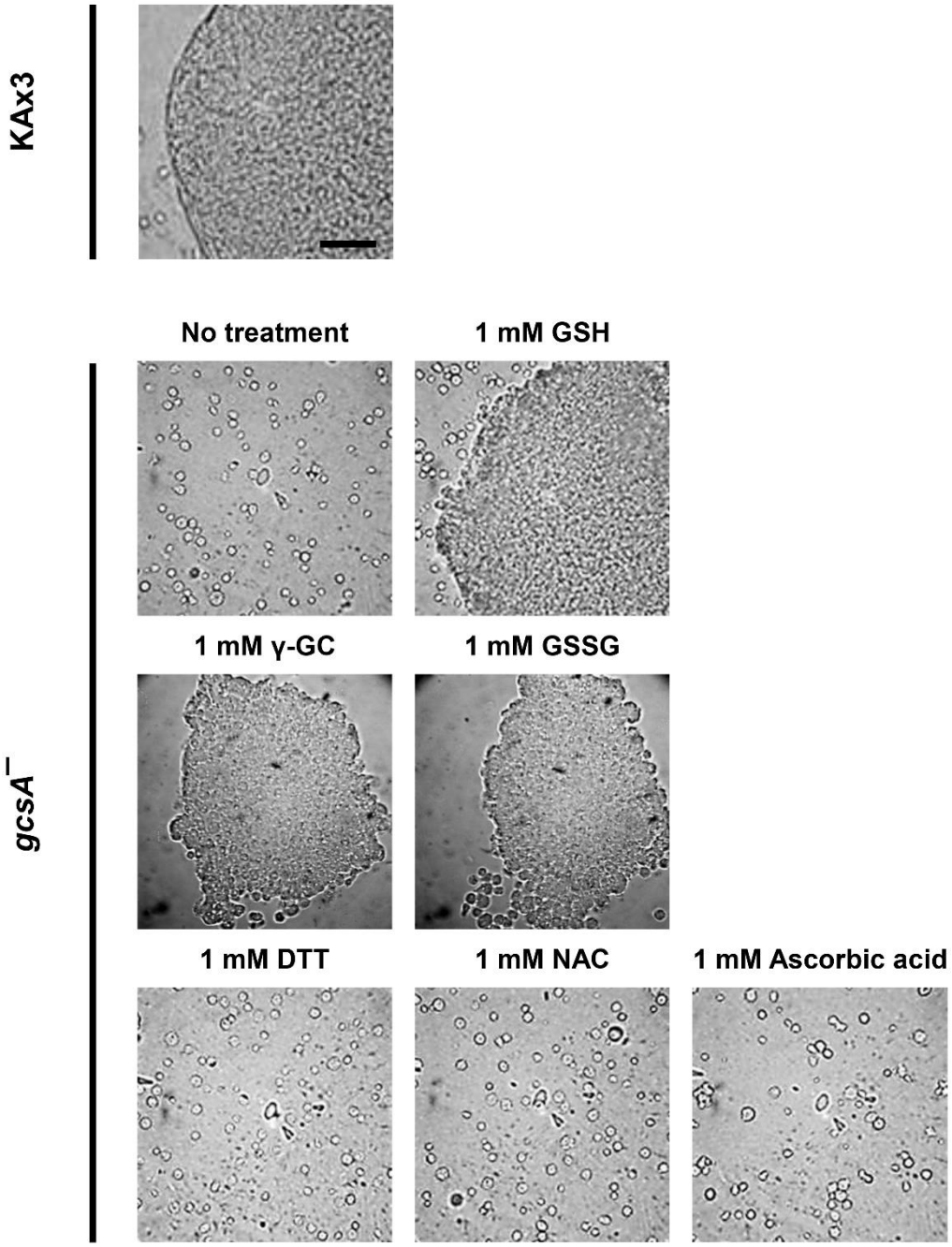

KAx3
No treatment
1 mM GSH
gcsA⁻
1 mM γ-GC
1 mM GSSG
1 mM DTT
1 mM NAC
1 mM Ascorbic acid

Fig. 8. Effect of other exogenous thiols or reducing agents on the development of KAx3 and *gcsA*$^{-}$ cells in suspension. KAx3 and *gcsA*$^{-}$ cells were subjected in non-nutrient KK2 buffer with 1 mM of γ-GC (γ-glutamyl cysteine), GSSG (oxidized glutathione), DTT (dithiothreitol), NAC (*N*-acetylcysteine), and ascorbic acid. The developmental morphology was observed at 12 h after development. The scale bar represents 0.05 mm.

potential is widely known. Depletion of GSH from cells may cause oxidative stress and lead to developmental defects in *Dictyostelium*. This possibility was examined that whether other thiol-containing compounds or reducing compounds rescued the developmental defects of *gcsA*$^{-}$ cells (Fig. 8). It was found that GSH-depleted *gcsA*$^{-}$ cells failed to form aggregates regardless of the concentration of thiol-containing molecule and a general reducing compound, such as dithiothreitol (DTT), *N*-acetylcysteine (NAC), and ascorbic acid. Only GSH induced the GSH-depleted *gcsA*$^{-}$ cells to initiate development. These results suggest that GSH plays indispensable roles independent of its redox properties in the initiation of *Dictyostelium* development.

2. Developmental properties of GSH-depleted *gcsA*$^{-}$ cells

It is known that the transcriptional expression of genes are regulated for developmental initiation in *Dictyostelium*. The expression of vegetative genes, which are most needed to maintain the growth and energy metabolism, decreases. But the expression of developmental genes, which are required for the proper progression of developmental life cycle, increases. To gain more information on the developmental status of *gcsA*$^{-}$ cells, the expression of genes that were needed to be regulated for optimal developmental initiation was monitored. KAx3 and *gcsA*$^{-}$ cells were allowed to develop in suspension and total RNA samples were prepared at various developmental times. The *cprD* expresses a cysteine protenase during growth but not during

development (Souza *et al.*, 1998). Northern blot analysis revealed that the expression level of *cprD* decreased in response to starvation signal and it was hard to detect at 4 h after development in KAx3 cells (Fig. 9). In contrast, the level of *cprD* remained high during development in *gcsA*$^{-}$ cells without the addition of GSH. When 1 mM GSH was added to *gcsA*$^{-}$ cells, the expression pattern of *cprD* was similar to that of KAx3 cells, although it was slightly delayed.

Further, the expression of *dscA* and *dia2* was observed. *dscA* and *dia2* are known as good markers for the transition from growth to development (Maeda, 2005), because their mRNA transcripts accumulate only during development. *dscA* and *dia2* were not expressed in GSH-depleted *gcsA*$^{-}$ cells (Fig. 10). However, the addition of 1 mM exogenous GSH induced the expression of them. The expression of *cprD, dscA*, and *dia2* seems to be modulated by GSH. These results indicate that GSH-depleted *gcsA*$^{-}$ cells are not ready to initiate developmental cycle.

3. The roles of GSH in the regulation of cAMP signaling

3.1. The expression of genes related with the cAMP signaling system in *gcsA*$^{-}$ cells

GSH depletion caused halt of life cycle progression from growth to development in *Dictyostelium*. GSH-depleted *gcsA*$^{-}$ cells did not form aggregates and existed as single cells. The cAMP signaling pathway is one of the earliest events to induce aggregation in multicellular development of *Dictyostelium* (Loomis, 1998). *carA* and *acaA*, encoding cAMP receptor cAR1 and adenylyl cyclase ACA, respectively, are key regulators of cAMP

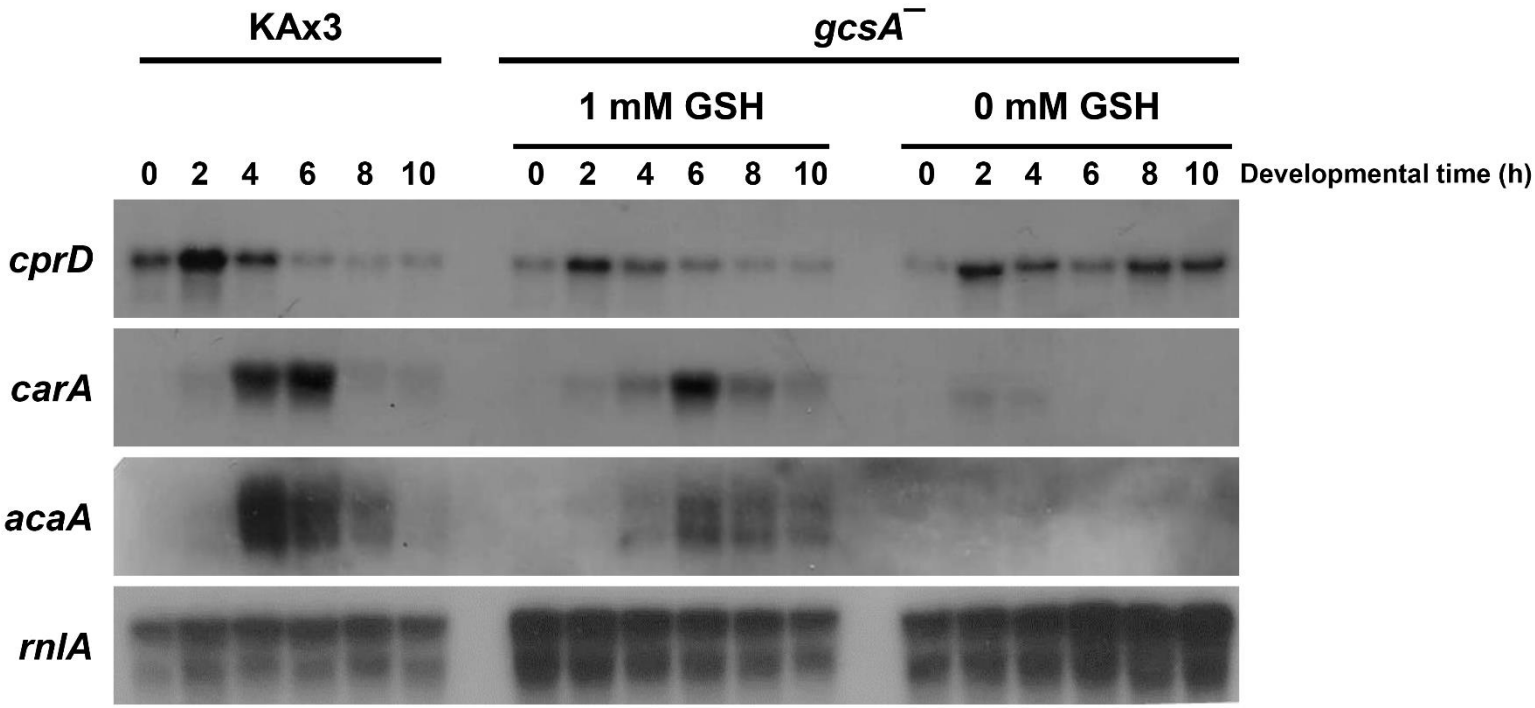


Fig. 9. Expression of early developmental genes in *gcsA*¯ cells during suspension development. KAx3 and *gcsA*¯ cells were allowed to develop in non-nutrient KK2 buffer for 10 h and the expression of genes, which were known to be regulated for the developmental initiation, was analyzed by Northern blotting. *cprD*, encoding vegetative-stage-specific serine proteinase; *carA*, encoding cAMP receptor 1; *acaA*, encoding adenylyl cyclase A; *rnlA*, a loading control.

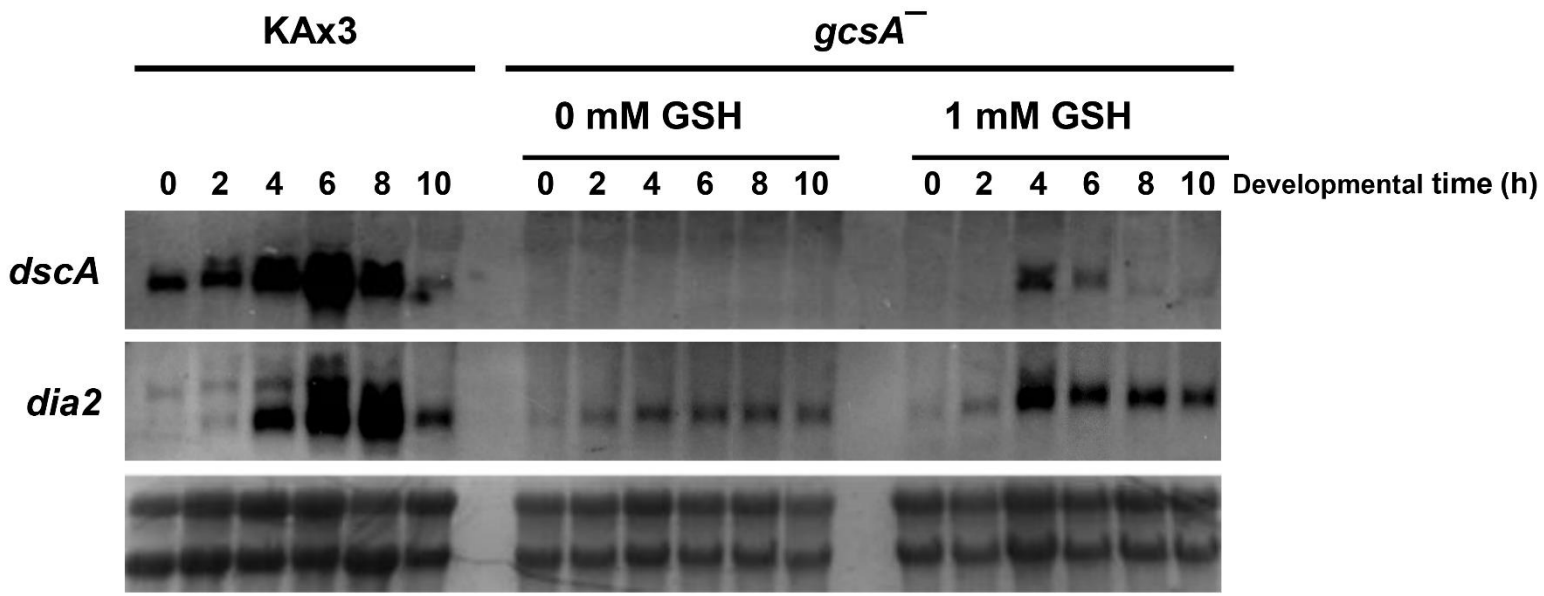


Fig. 10. Expression of *dscA* and *dia2* in *gcsA*$^{-}$ cells during suspension development. KAx3 and in *gcsA*$^{-}$ cells were allowed to develop in non-nutrient KK2 buffer. Developmental stage of in *gcsA*$^{-}$ cells in suspension was confirmed by analyzing the expression of *dscA* and *dia2*, which serves as markers for the initiation of development. *dscA*, encoding discoidin A; *dia2*, encoding novel lysine- and leucine-rich protein (DIA2).

signaling. The regulation of the expression of *carA* and *acaA* is required to initiate development (Anjard et al., 1992; Klein et al., 1988; Mann et al., 1992; Pitt et al., 1992). To investigate the cause of the developmental defects of GSH-depleted *gcsA*$^{-}$ cells, the expression of *carA* and *acaA* was examined by Northern blot analysis (Fig. 9). KAx3 and *gcsA*$^{-}$ cells were allowed to develop in suspension and total RNA samples were prepared at every 2 h intervals. The results showed that the expression of *carA* and *acaA* was induced in KAx3 cells. In contrast, the expression levels of *carA* and *acaA* were undetectably low in *gcsA*$^{-}$ cells in the absence of added GSH during the entire time suspension development. When 1 mM GSH was added to *gcsA*$^{-}$ cells, the expression pattern of *carA* and *acaA* was similar to that of KAx3 cells, although it was slightly decreased and delayed. These results indicate that GSH induces development through activating the expression of early developmental genes, particularly those involved in cAMP signaling in *Dictyostelium*.

3.2. The effect of cAMP stimulation on development of *gcsA*$^{-}$ cells

GSH-depleted *gcsA*$^{-}$ cells showed lack of gene expression related with the cAMP signaling cascade. Expression of ACA is one of the earliest responses of cells to starvation. This suggests that the absence cAMP signaling causes the aggregate-less phenotype of GSH-depleted *gcsA*$^{-}$ cells. The deficiency of cAMP oscillations because of the absence of ACA might be the defect in *gcsA*$^{-}$ cells. Insall *et al.* (1994) reported that that some aggregation-deficient mutants form aggregates and induce the expression of cAMP response genes when they are periodically stimulated with exogenous

cAMP. Thus, it was examined whether exogenously added cAMP pulses rescued the developmental defects of *gcsA*$^{-}$ cells. KAx3 and *gcsA*$^{-}$ cells were allowed to develop in suspension with nanomolar concentration of cAMP as described in materials and methods. According to the results, cAMP stimulation activates and accelerates formation of aggregates in both KAx3 and *gcsA*$^{-}$ cells with 1 mM GSH (Fig. 11). The aggregates were bigger and tighter than cAMP untreated cells, which are shown in Fig. 6. However, *gcsA*$^{-}$ cells were remained as single cells without the addition of GSH in spite of exogenously added cAMP pulses. For detailed analysis of developmental morphology, the progress of aggregation was observed by developmental time. *gcsA*$^{-}$ cells showed slightly late progression rate of aggregates formation by a few hours in comparison with KAx3 cells when they were subjected to non-nutrient buffer with 1 mM GSH (Fig. 12). Without GSH, *gcsA*$^{-}$ cells were in single cell state for 14 h in spite of the supplementation of cAMP pulses. In addition, the expression of *carA* and *acaA* was induced by exogenous cAMP stimulation in GSH-depleted *gcsA*$^{-}$ cells (data not shown). These results suggest that lack of cAMP secretion is not a main cause of aggregate-less phenotype of the GSH-depleted *gcsA*$^{-}$ cells.

3.3. The effect of cAR1 expression on development of *gcsA*$^{-}$ cells

Developmental defects of *gcsA*$^{-}$ cells were not explained by the absence of cAMP synthesis and secretion, because *gcsA*$^{-}$ cells did not develop and failed to induce the expression of *carA* and *acaA* without GSH even though exogenous cAMP was added periodically (Figs. 11 and 12).

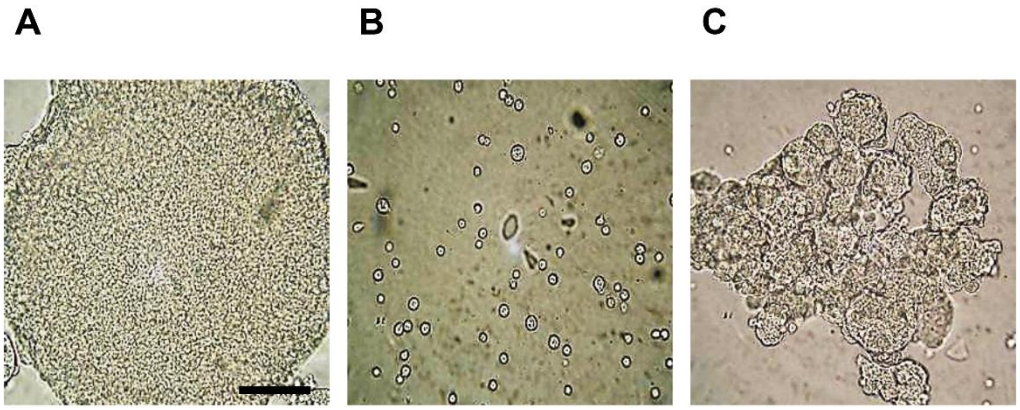


Fig. 11. Developmental morphology of KAx3 and *gcsA*$^{-}$ cells in suspension with cAMP pulses. KAx3 and *gcsA*$^{-}$ cells were allowed to develop in non-nutrient KK2 buffer with pulsed addition of nanomolar levels of cAMP and photographed at 12 h. (A) KAx3 cells, (B) *gcsA*$^{-}$ cells with no GSH, (C) *gcsA*$^{-}$ cells with 1 mM GSH. The scale bar represents 0.05 mm.

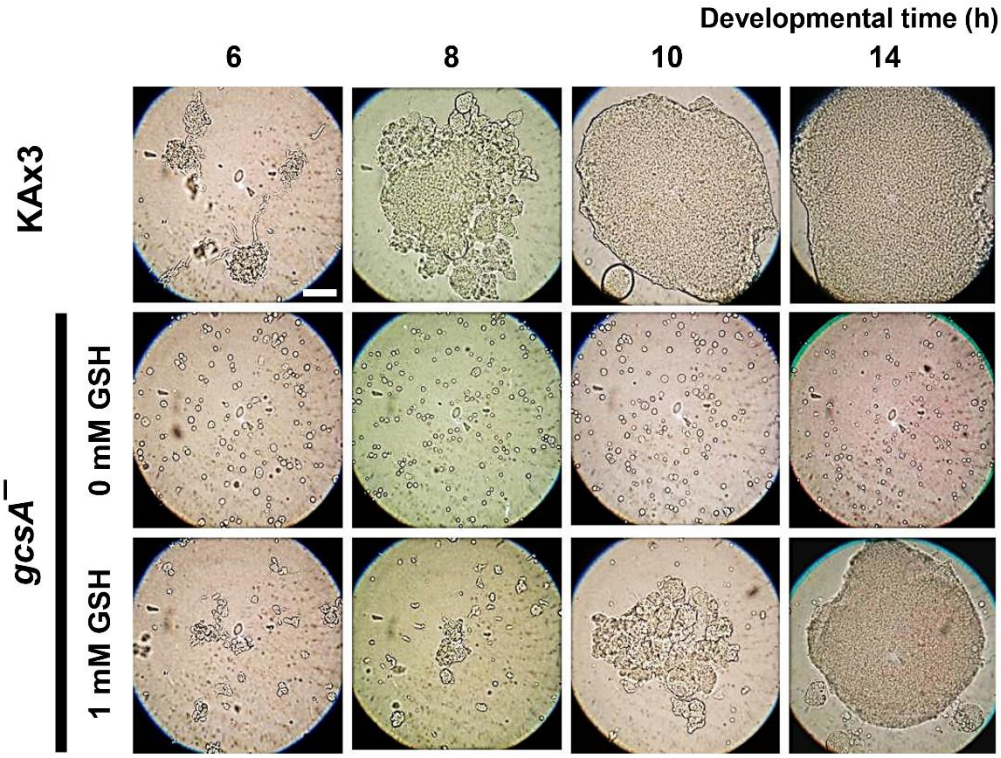


Fig. 12. Developmental morphology of KAx3 and *gcsA*¯ cells in suspension. KAx3 and *gcsA*¯ cells were allowed to develop in non-nutrient KK2 buffer with nanomolar levels of cAMP pulses. The developmental morphology was observed and photographed in time at 2 h intervals. The scale bar represents 0.05 mm.

Next, other possibility was considered that the failure of *gcsA*$^{-}$ cells to develop is reasoned by the defect in cAMP recognition. The cAMP receptor is required to induce development of *Dictyostelium*. Binding of cAMP to cAR1 is required for the activation of several second-messenger pathways, including G-protein-independent stimulation of calcium uptake, and G-protein dependent stimulation of adenylyl and guanylyl cyclases (Kesbeke *et al.*, 1988; Kumagai *et al.*, 1989; Milne and Coukell, 1991; Milne and Devreotes, 1993; Pupillo *et al.*, 1992; Sun *et al.*, 1990). As shown in Northern blotting analysis, *carA* encoding cAMP receptor (cAR1) was not expressed in *gcsA*$^{-}$ cells without the addition of 1 mM GSH (Fig. 9). Moreover, exogenous cAMP pulses failed to induce the expression of *carA* and failed to rescue the developmental defect of *gcsA*$^{-}$ cells. Thus, it was suspected that the failure of development in *gcsA*$^{-}$ might be due to the lack of cAR1 or deficiency of cAR1 activation.

It was determined whether constitutive expression of cAR1 under control of an actin promoter in *gcsA*$^{-}$ cells reversed the defects caused by depletion of GSH. Full-length of cAR1 was cloned into an integrating expression vector Exp4(+) under the control of *Actin15* promoter and the *act15::carA* expression construct was introduced to KAx3 and *gcsA*$^{-}$ cells (Fig. 13A). And the induced expression of cAR1 in both KAx3 and *gcsA*$^{-}$ cells was confirmed by Northern blotting (Fig. 13B). Developmental phenotype of cAR1-expressing KAx3 (cAR1^{OE}/KAx3) and *gcsA*$^{-}$ cells (cAR1^{OE}/*gcsA*$^{-}$) was observed when they were allowed to develop in suspension with cAMP pulses (Fig. 14). Although cAR1 was expressed, cAR1^{OE}/*gcsA*$^{-}$ cells did not develop in the absence of added GSH. The failure of *gcsA*$^{-}$ cells to develop was not attributed to the inability of cAR1

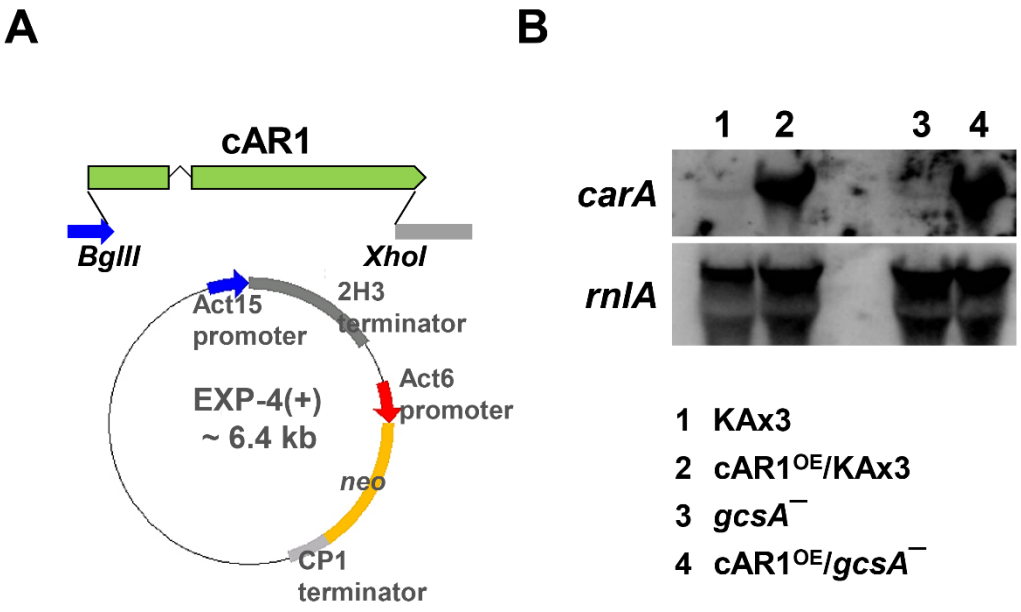


Fig. 13. Constitutive expression of cAR1 in KAx3 and *gcsA*$^{-}$ cells. (A) Preparation of the construct for cAR1 expression. Full-length of gDNA was cloned into integrating expression Exp4(+) vector containing constitutive *Act15* promoter. (B) Confirmation of cAR1 expression in KAx3 and *gcsA*$^{-}$ cells. The cAR1 expression in KAx3 and *gcsA*$^{-}$ cells was confirmed by analyzing the expression levels of *carA* mRNA by Northern blotting. *carA*, encoding cAMP receptor; *rnlA*, a loading control.

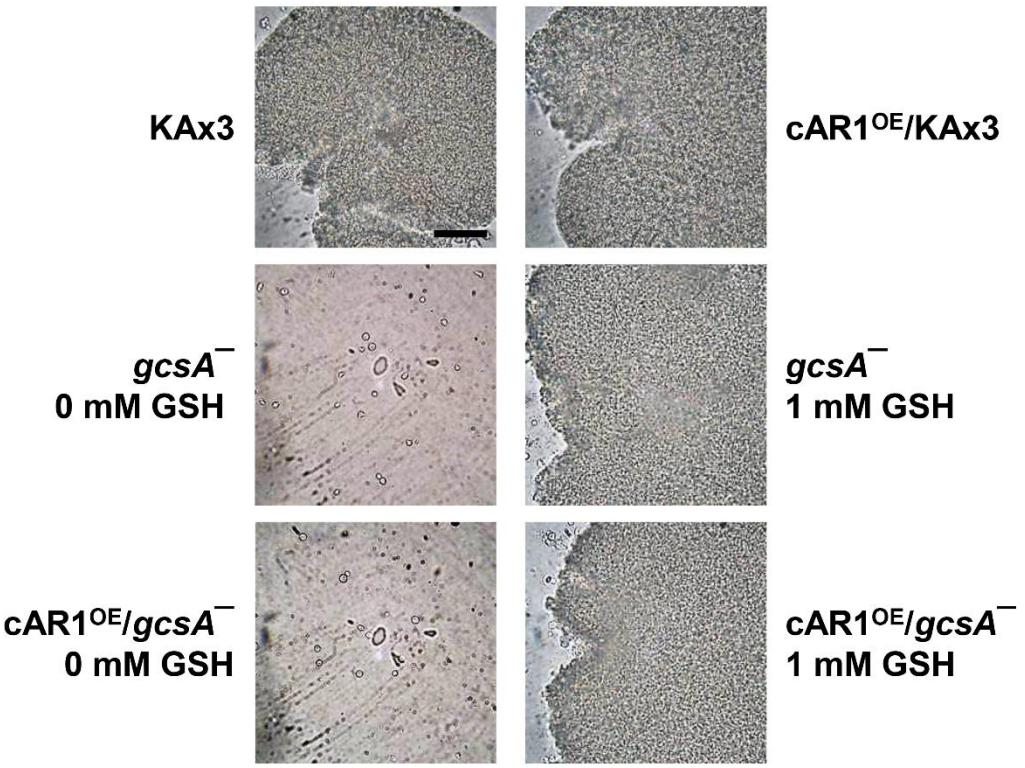


Fig. 14. Effect of cAR1 expression on the developmental morphology of *gcsA*$^{-}$ cells in suspension. cAR1-expressing KAx3 and *gcsA*$^{-}$ cells (cAR1^{OE}/KAx3 and cAR1^{OE}/*gcsA*$^{-}$ cells, respectively) were allowed to develop in non-nutrient KK2 buffer with cAMP pulses and photographed at 12h. The scale bar represents 0.05mm.

to respond to cAMP.

To determine whether the developmental defect in cAR1^{OE}/*gcsA*$^{-}$ cells was due to a deficiency in other components of the cAMP signaling pathway, the expression levels of *carA*, *acaA*, *gpaB* (G-protein alpha subunit 2), *pkaC*, and *pkaR* were determined. It was found that the expression of *acaA* and *gpaB* was induced by the constitutive expression of *carA* in *gcsA*$^{-}$ cells (cAR1^{OE}/*gcsA*$^{-}$) without GSH (Fig. 15). However, the transcriptional expression of *pkaC* and *pkaR* was not affected significantly by the constitutive expression of cAR1. In other words, *gcsA*$^{-}$ cells did not form aggregates in the absence of GSH when cAR1 was expressed, although cAMP signaling was activated at a functional level. These results suggest that GSH interacts with other pathway which functions at earlier step than cAMP signaling to regulate the transition from growth to development.

4. The role of GSH in the regulation of YakA signaling

4.1. The expression of *yakA* in *gcsA*$^{-}$ cells

gcsA^{-} cells showed aggregate-less phenotype and abnormal transcriptional regulation of early developmental genes, especially *carA* and *acaA* which are important components of the cAMP signaling system. And the restoration of *carA* and *acaA* expression by cAMP stimulation and cAR1 expression did not rescue the developmental defect of *gcsA*$^{-}$ cells. Activation of the YakA signaling pathway is known the earliest developmental regulatory event before cAMP signaling occurs. When cells are starved, YakA inhibits the expression of vegetative-state-specific gene, in particular

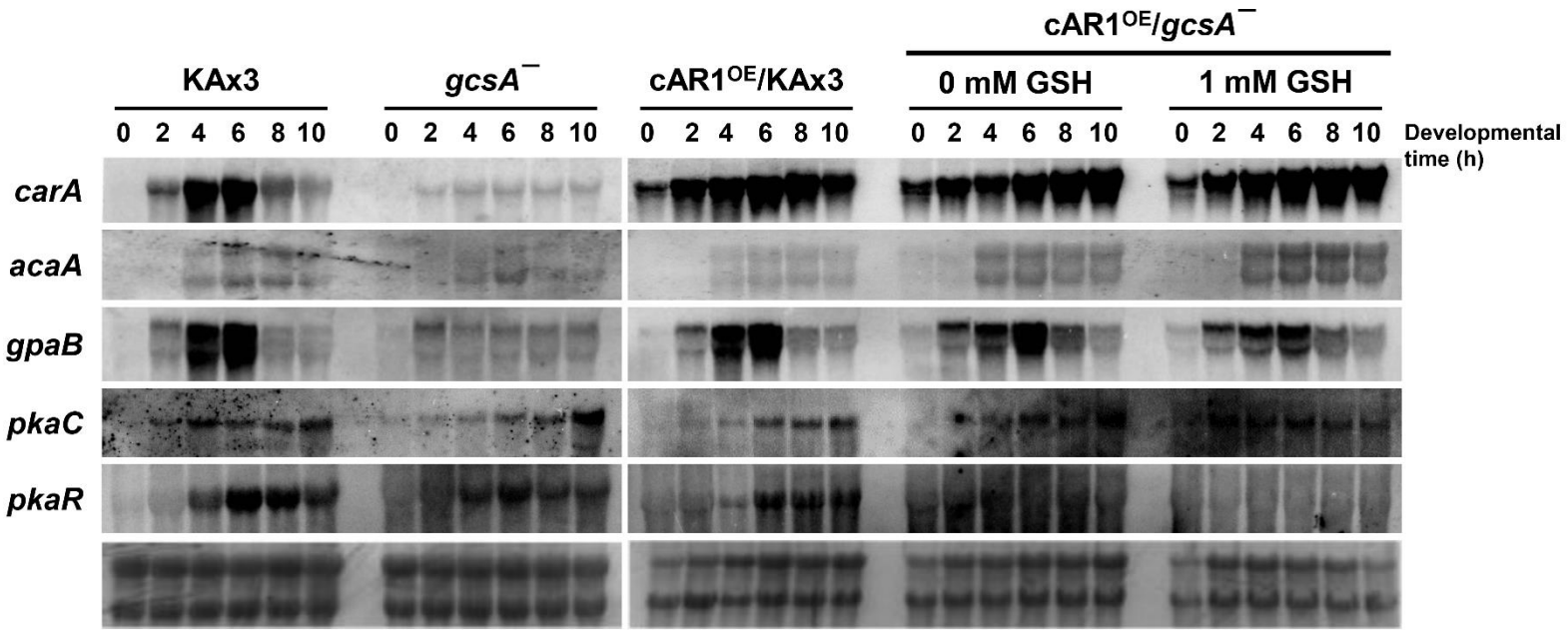


Fig. 15. Effect of cAR1 expression on developmental gene expression. KAx3, *gcsA*$^{-}$, cAR1^{OE}/KAx3, and cAR1^{OE}/*gcsA*$^{-}$ cells were allowed to develop in non-nutrient KK2 buffer with cAMP pulses. The expression of genes related to the cAMP signaling pathway was analyzed by Northern blotting. *carA*, encoding cAMP receptor 1; *acaA*, encoding adenylyl cyclase A; *gpaB*, encoding G-protein alpha subunit 2; *pkaC*, encoding cAMP-dependent protein kinase A catalytic subunit; *pkaR*, encoding cAMP-dependent protein kinase A regulatory subunit.

to reduce *pufA* expression, which inhibits translation of *pkaC* mRNA (Souza et al., 1999) and induces an increase in the expression of aggregation-state-specific genes, such as *carA* and *acaA* (Souza et al., 1998).

To investigate the relationship between GSH and the YakA, the expression of *yakA* was determined in *gcsA*$^{-}$ cells. The transcription of *yakA* was induced as the nutrient exhausted and reach a maximum at 6 h, and after then fell slowly in KAx3 cells (Fig. 16). The level of *yakA* expression in the *gcsA*$^{-}$ cells remained very low during development in suspension without the addition of GSH, which was similar levels to that of *yakA*$^{-}$ cells. Interestingly, the expression of *yakA* was induced by adding 1 mM GSH to *gcsA*$^{-}$ cells; however, the levels were slightly lower than those in KAx3 cells. These findings indicate that the expression of *yakA* may be regulated by the intracellular GSH.

4.2. The effect of intracellular GSH on the expression of *yakA*

According to above results, the expression of *yakA* seems to be regulated by intracellular GSH to initiate development of *Dictyostelium*. These results were confirmed by analyzing the expression of *yakA* in KAx3 cells when exposed to diverse concentration of GSH. KAx3 cells were allowed to develop with 0 mM, 0.5 mM, 1.0 mM, 2.0 mM and 3.0 mM of GSH in non-nutrient KK2 buffer. As the concentration of GSH increased, the expression of *yakA* increased proportionally and reached a peak more rapidly compared to that with the control (Fig. 17). Aggregation started slightly faster in proportion to the concentration of GSH (Fig. 18). Exogenously added GSH induced the expression of *yakA*, and to demonstrate these results

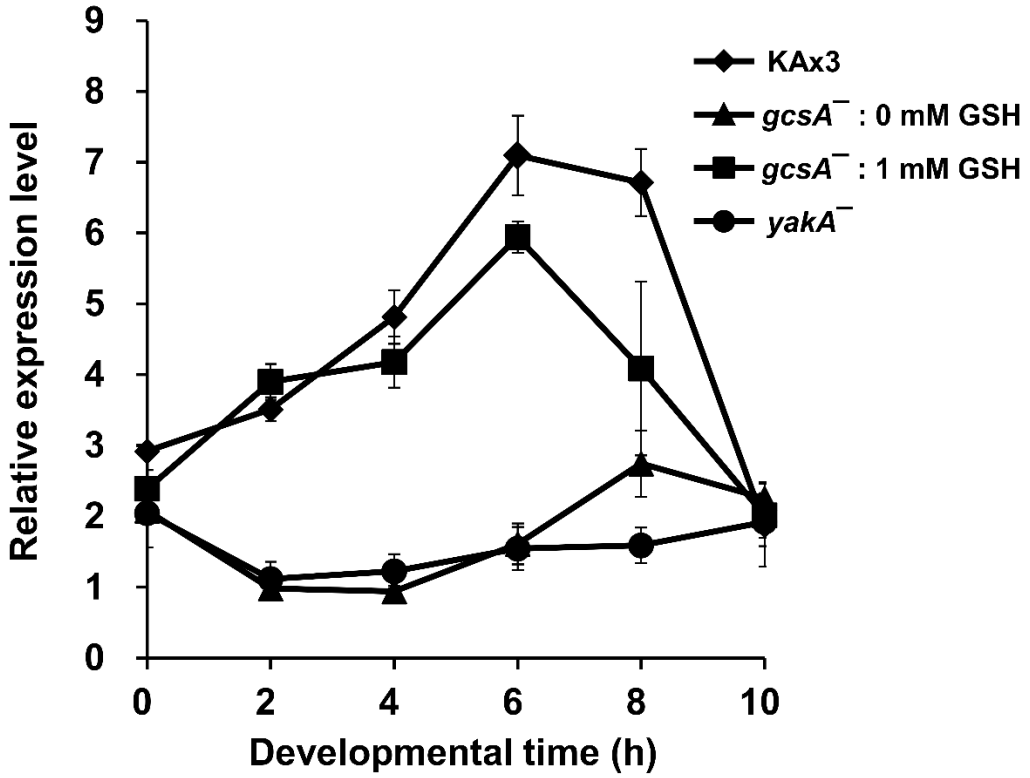


Fig. 16. Expression levels of *yakA* in KAx3 and *gcsA*$^{-}$ cells during development in suspension. KAx3, *gcsA*$^{-}$, and *yakA*$^{-}$ cells were allowed to develop in non-nutrient KK2 buffer for 10 h, and total RNA was extracted at 2 h intervals. The expression of *yakA* was analyzed using real-time RT-PCR. All expression data were normalized by dividing the amount of *yakA* by the amount of *rnlA* used as a control. The values represent mean ± S.E.M. of three independent experiments. *yakA*, protein serine/threonine kinase.

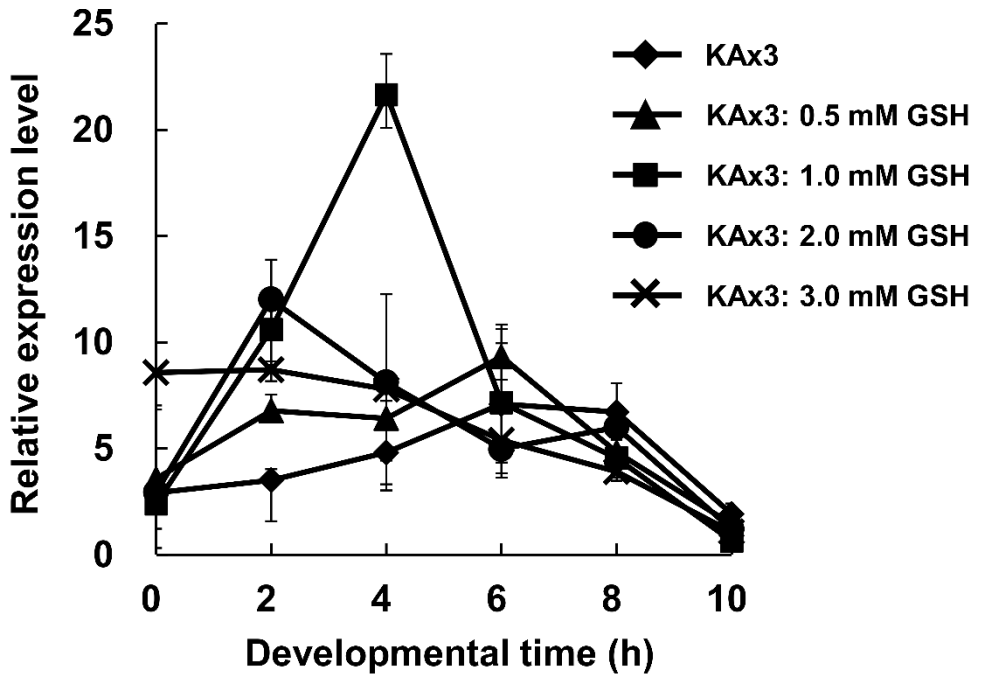


Fig. 17. Effect of exogenous GSH on the expression patterns of *yakA* during development in suspension. KAx3 cells were allowed to develop in non-nutrient KK2 buffer for 10 h with diverse concentration of GSH; 0 mM, 0.5 mM, 1.0 mM, 2.0 mM, and 3.0 mM of GSH. Total RNA was extracted at 2 h intervals. The expression of *yakA* was analyzed using real-time RT-PCR. All expression data were normalized by dividing the amount of *yakA* by the amount of *rnlA* used as a control. The values represent mean ± S.E.M. of three independent experiments. *yakA*, protein serine/threonine kinase.

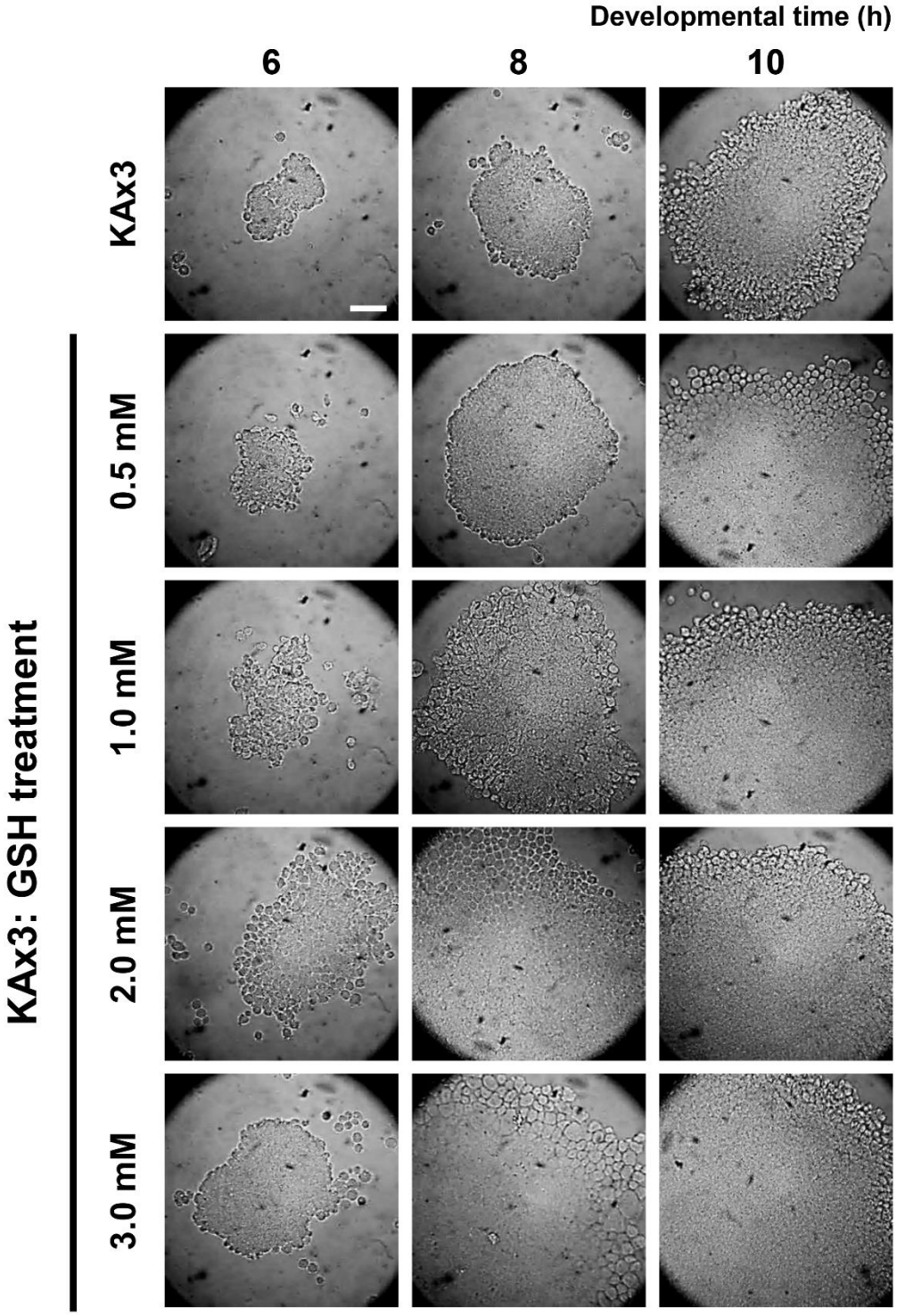

Developmental time (h)
6
8
10
KAx3
KAx3: GSH treatment
0.5 mM
1.0 mM
2.0 mM
3.0 mM

Fig. 18. Effect of exogenous GSH on the progress of the formation of aggregates. KAx3 cells were allowed to develop in non-nutrient KK2 buffer for 10 h in the presence of 0 mM, 0.5 mM, 1.0 mM, 2.0 mM, and 3.0 mM GSH and photographed at the indicated time. The scale bar represents 0.05 mm.

further, the expression of *yakA* and developmental morphology were observed in GCS-overexpressing KAx3 cells (GCS^{OE}/KAx3). GCS^{OE}/KAx3 cells showed significantly increased intrinsic GSH contents more than 160% compared to that of KAx3 cells (Fig. 19). The same events as the *yakA* expression and developmental morphology in KAx3 cells treated with exogenous GSH occurred in GCS^{OE}/KAx3. The expression of *yakA* was considerably increased and reached the peak 2 h earlier than that of KAx3 cells (Fig. 20). The formation of aggregates also occurred at a slightly faster rate than in KAx3 cells (Fig. 21). These results indicate that GSH regulates the transition from growth to development by regulating the expression of *yakA*.

4.3. The expression of YakA downstream regulators in *gcsA*$^{-}$ cells

yakA was not expressed appropriately without GSH. It was assumed that diminished expression of *yakA* caused blocked developmental life cycle in *gcsA*$^{-}$ cells. YakA signaling system is composed of YakA, PufA, PKA, and ACA. The expression of downstream regulators of YakA signaling cascade was also monitored.

4.3.1. The expression of *pufA*

First, the expression of *pufA* was analyzed by Northern blotting. PufA is a translational inhibitor of PKA-C and transcription of *pufA* is regulated by YakA (Souza *et al.*, 1999). Increased YakA during development inhibits its transcription and eventually resulted in the increased PKA activity. The results showed that the expression of *pufA* increased in GSH-depleted *gcsA*$^{-}$

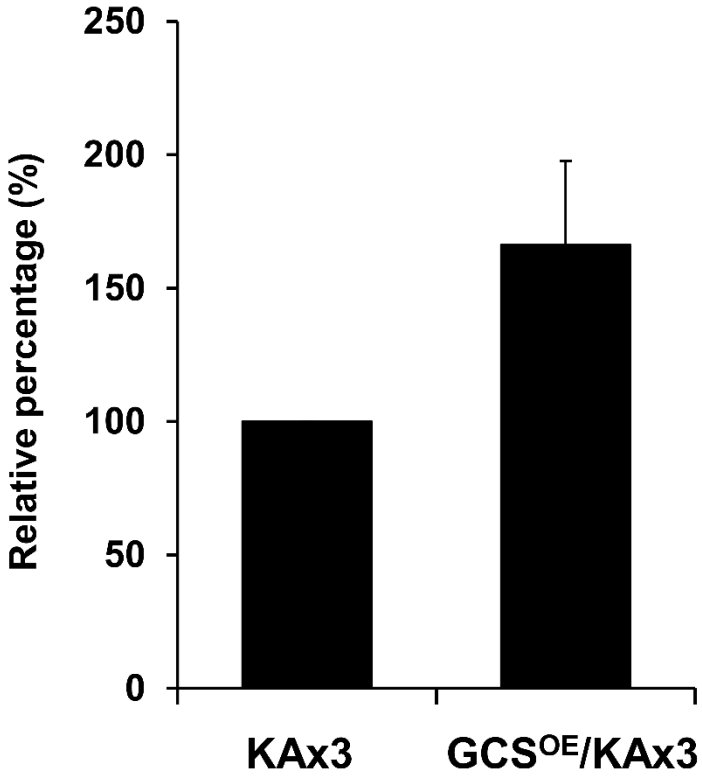


Fig. 19. Intracellular GSH concentration of GCS^{OE}/KAx3 cells. The concentration of intracellular GSH was measured in exponentially growing KAx3 and GCS^{OE}/KAx3 cells using HPLC and fluorescent detector. Intracellular GSH was modified to a mBBr-conjugated form to detect. The concentration of GSH was calculated in relative values compared to that of KAx3 cells. The values represent the mean ± S.E.M. of three independent experiments.

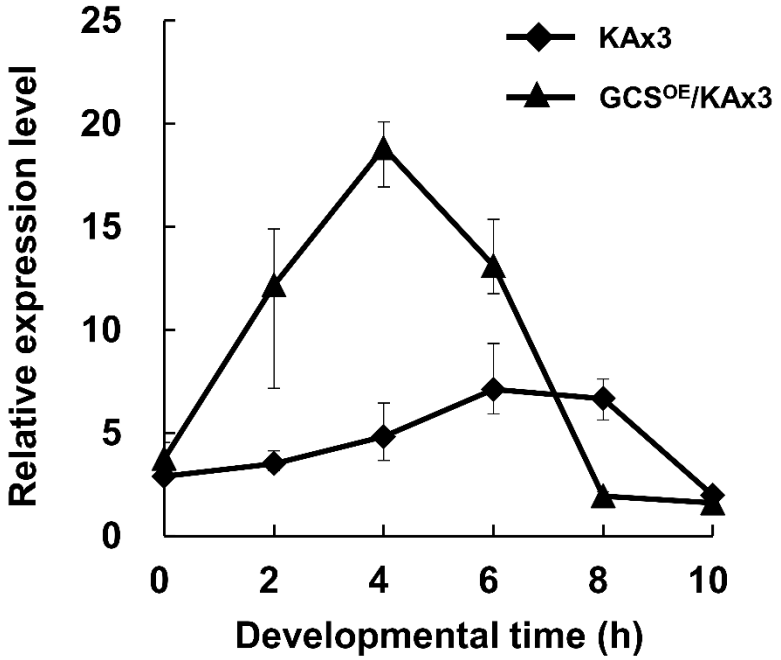


Fig. 20. Effect of the constitutive expression of GCS in KAx3 cells on the expression patterns of *yakA* during development in suspension. KAx3 and GCS^{OE}/KAx3 cells were allowed to develop in non-nutrient KK2 buffer for 10 h and total RNA was extracted at 2 h intervals. The expression of *yakA* was analyzed using real-time RT-PCR. All expression data were normalized by dividing the amount of *yakA* by the amount of *rnlA* used as a control. The values represent the mean ± S.E.M. of three independent experiments. *yakA*, protein serine/threonine kinase.

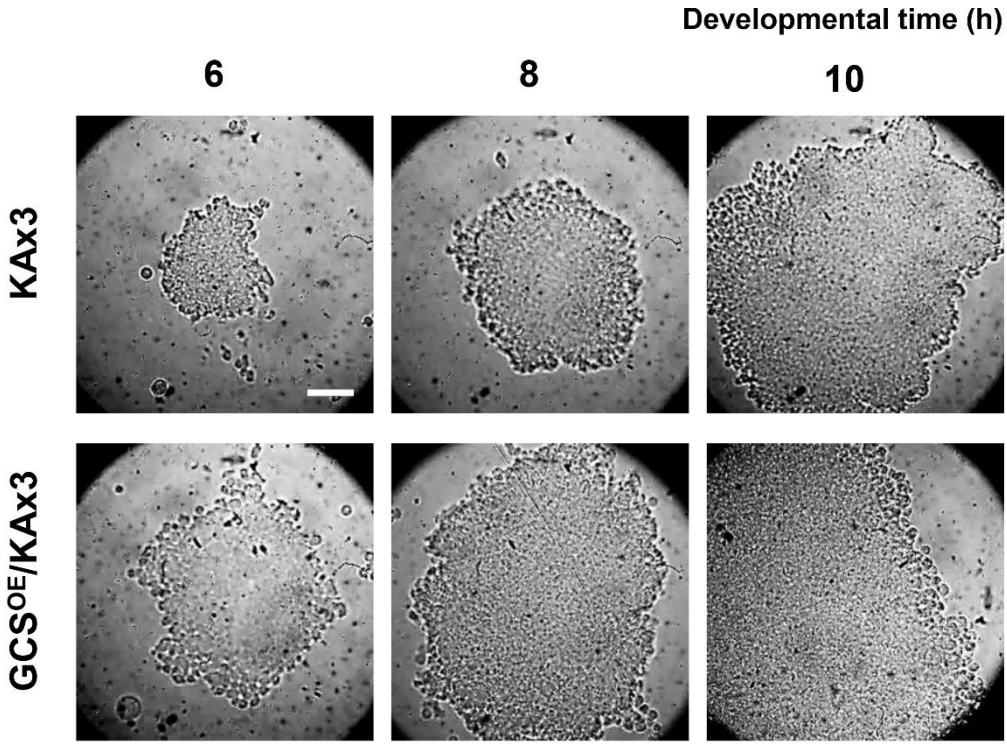


Fig. 21. Developmental morphology of GCS^{OE}/KAx3 cells in suspension. KAx3 and GCS^{OE}/KAx3 cells were allowed to develop in non-nutrient KK2 buffer with cAMP pulses for 10 h and photographed at the indicated time. The scale bar represents 0.05 mm.

cells and the supplementation of GSH repressed the expression of *pufA* in *gcsA*$^{-}$ cells (Fig. 22). These results are consistent with the lowered *yakA* expression in *gcsA*$^{-}$ cells.

4.3.2. The gene expression and the enzymatic activity of PKA in *gcsA*$^{-}$ cells

Next, transcriptional expression of *pkaC* and the enzymatic activity of PKA were determined in *gcsA*$^{-}$ cells because the expression of *pufA*, which inhibits PKA-C translation, increased in *gcsA*$^{-}$ cells (Fig. 22). According to the Northern blotting results, the expression of *pkaC*, a catalytic subunit of PKA, was not extensively affected by intracellular GSH (Fig. 22). *pkaC* was expressed in *gcsA*$^{-}$ cells without GSH with similar level to KAx3 cells and *gcsA*$^{-}$ cells with 1 mM GSH. However, PKA activity was much lower in *gcsA*$^{-}$ cells without the addition of GSH than KAx3 cells (Fig. 23). In contrast, the activity of PKA recovered to similar levels compared with KAx3 cells when 1 mM GSH was added. The lowered activity of PKA in GSH-depleted *gcsA*$^{-}$ cells was consistent with the decreased *yakA* expression and the increased *pufA* expression shown in Figs. 16 and 21, respectively. These results indicate that intracellular GSH is needed to activate the YakA signaling pathway which is required for the transition from growth to development.

5. Developmental properties of *yakA*$^{-}$ cells

5.1. The developmental morphology of *yakA*$^{-}$ cells

To explain the relation between YakA and GSH more, developmental

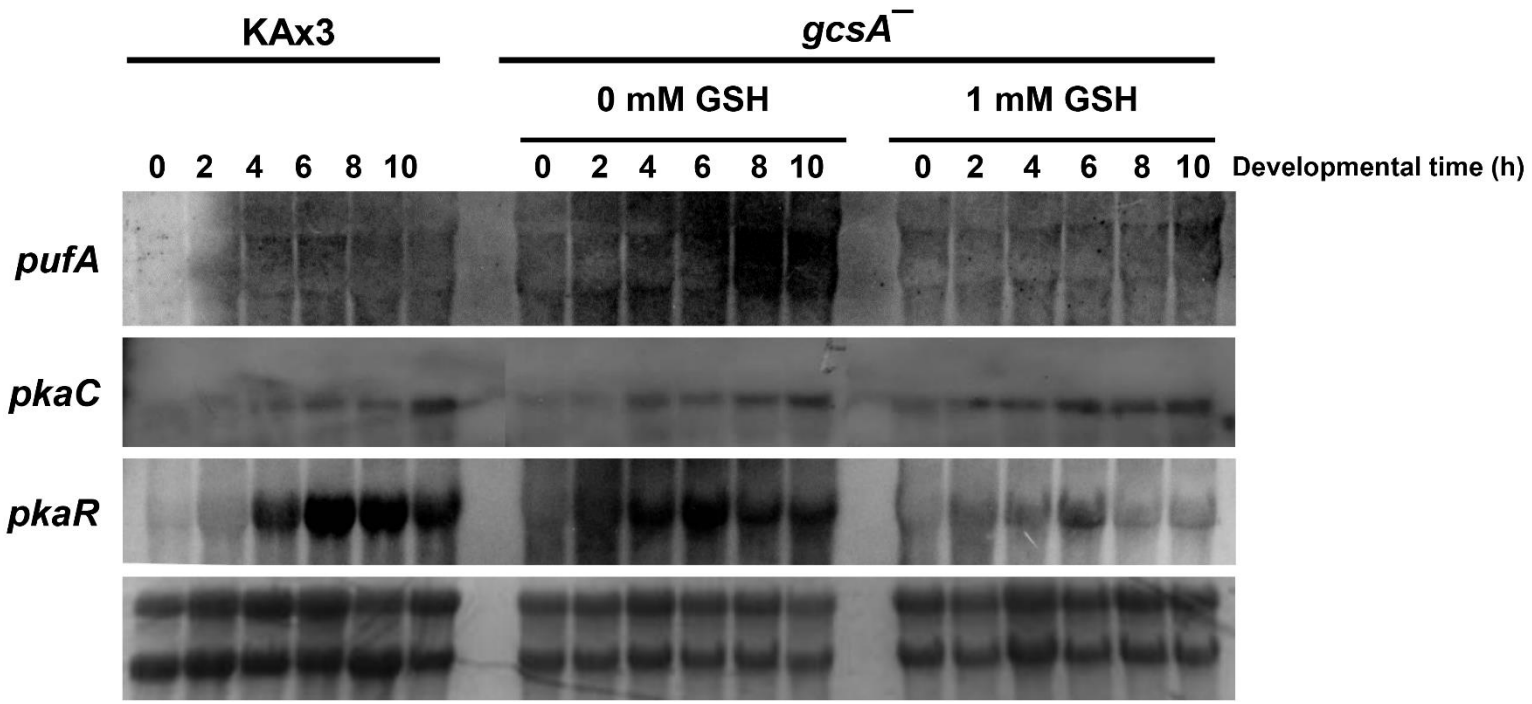


Fig. 22. Expression of downstream regulators of the YakA signaling system in KAx3 and *gcsA*¯ cells. KAx3 and *gcsA*¯ cells were allowed to develop in non-nutrient KK2 buffer with cAMP pulses. The expression of genes encoding downstream regulators of the YakA signaling pathway was analyzed by Northern blotting. *pufA*, encoding a RNA binding protein; *pkaC*, encoding protein kinase catalytic subunit; *pkaR*, encoding protein kinase regulatory subunit.

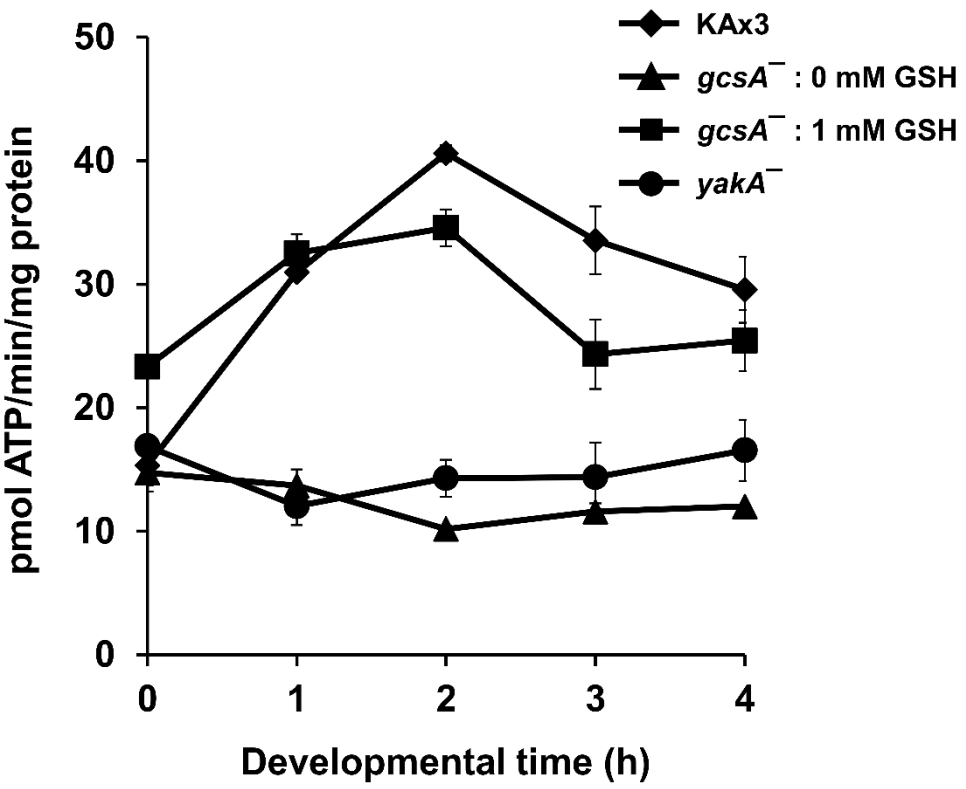


Fig. 23. PKA activity in KAx3, *gcsA*¯, and *yakA*¯ cells during development in suspension. KAx3, *gcsA*¯, and *yakA*¯ cells were allowed to develop in non-nutrient KK2 buffer with cAMP pulses. The activity of PKA was measured using the SignaTECT PKA Activity System (Promega). The values represent the mean ± S.E.M. of three independent experiments.

morphology of *yakA*$^{-}$ cells was observed. As previously reported by Souza *et al.* (1998), *yakA*$^{-}$ cells were completely deficient in the formation of aggregation when they were placed on non-nutrient agar plates (data not shown). To analyze the developmental state of *yakA*$^{-}$ cells, *yakA*$^{-}$ cells were induced to develop in suspension. When suspended in non-nutrient KK2 buffer, they existed as single cells as *gcsA*$^{-}$ cells (Fig. 24). These results suggest that *gcsA*$^{-}$ cells and *yakA*$^{-}$ could not develop because they do not express *yakA* in response to developmental environment.

5.2. The expression of developmental genes in *yakA*$^{-}$ cells

Interestingly, *yakA*$^{-}$ cells showed remarkably similar patterns of early developmental gene expression and PKA activity compared with *gcsA*$^{-}$ cells. The expression of *carA*, *acaA*, and *pkaR* decreased significantly. In contrast, the expression of *pufA* increased significantly in GSH-depleted *gcsA*$^{-}$ and *yakA*$^{-}$ cells compared with KAx3 cells (Fig. 25). The activity of PKA was also decreased in both *gcsA*$^{-}$ and *yakA*$^{-}$ cells (Fig. 23). These results show that low expression levels of *yakA* and its downstream regulators and developmental defects are found in both *gcsA*$^{-}$ and *yakA*$^{-}$. Taken together, these results suggest that the developmental defects of *gcsA*$^{-}$ cells are caused by the lack of *yakA* expression which is essential for the initiation of development by activating PKA and triggers the expression of *carA* and *acaA*.

5.3. The effect of GSH on the developmental morphology of *yakA*$^{-}$ cells

It was examined that whether the supplementation of GSH rescued the

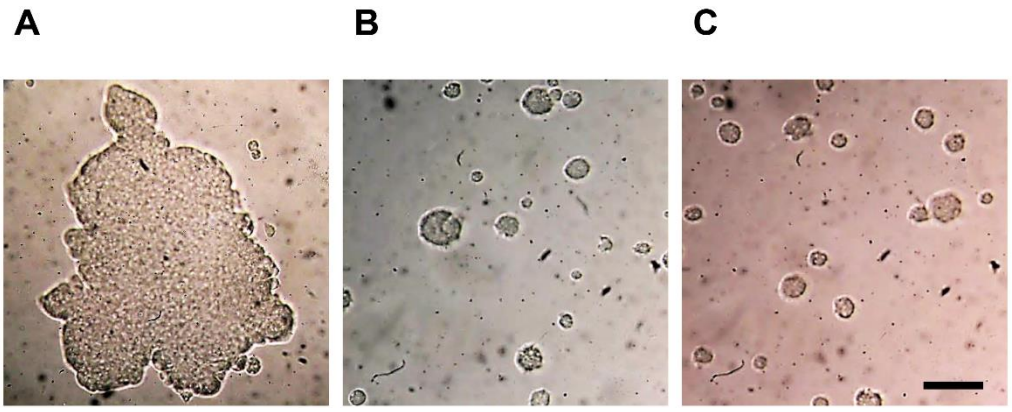


Fig. 24. Developmental morphology of *yakA*$^{-}$ cells in suspension. KAx3 and *yakA*$^{-}$ cells were allowed to develop in non-nutrient KK2 buffer with periodically added cAMP pulses and photographed at 12 h. (A) KAx3 cells, (B) *yakA*$^{-}$ cells, (C) *gcsA*$^{-}$ cells with no GSH. The scale bar represents 0.05 mm.

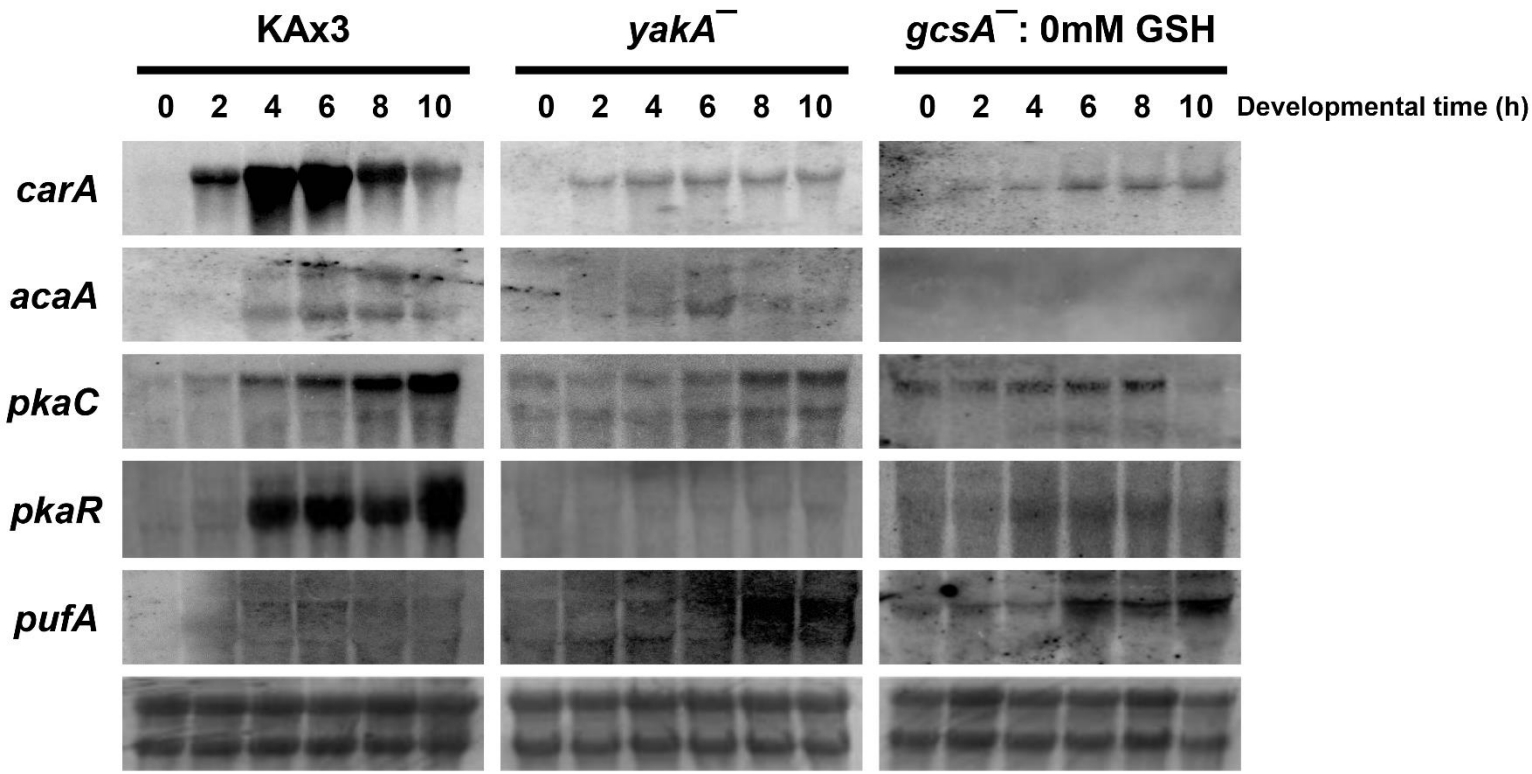

KAx3
yakA¯
gcsA¯: 0mM GSH
0 2 4 6 8 10
0 2 4 6 8 10
0 2 4 6 8 10
Developmental time (h)
carA
acaA
pkaC
pkaR
pufA

Fig. 25. Expression of developmental genes in *yakA*$^{-}$ cells. . KAx3, *gcsA*$^{-}$, and *yakA*$^{-}$ cells were allowed to develop in non-nutrient KK2 buffer. The expression patterns of several developmental genes were analyzed in *yakA*$^{-}$ cells by Northern blotting and compared with the expressions of KAx3 and *gcsA*$^{-}$ cells. *carA*, encoding cAMP receptor 1; *acaA*, encoding adenylyl cyclase A; *pkaC*, encoding protein kinase catalytic subunit; *pkaR*, encoding protein kinase regulatory subunit; *pufA*, encoding a RNA binding protein.

developmental defect of *yakA*$^{-}$ cells as did in *gcsA*$^{-}$ cells. *yakA*$^{-}$ cells were induced to develop with cAMP pulses and 1 mM GSH in non-nutrient KK2 buffer. They did not develop and existed as single cells though GSH was added (Fig. 26). These results imply that GSH regulates the initiation of development by activating the expression of *yakA* in *Dictyostelium*.

6. The role of GSH in the regulation of YakA signaling

6.1. The effect of YakA expression on the developmental morphology of *gcsA*$^{-}$ cells

The results so far achieved propose that GSH obviously has role in the life cycle shift from growth to development in *Dictyostelium* by regulating the expression of *yakA* and the aggregate-less phenotype of *gcsA*$^{-}$ cells is explained by the absence of *yakA* expression. To support these results, it was investigated whether constitutive expression of YakA could restore the defects in *gcsA*$^{-}$ cells. YakA was continuously expressed under the control of the actin15 promoter in KAx3 (YakA^{OE}/KAx3) and *gcsA*$^{-}$ (YakA^{OE}/*gcsA*$^{-}$) cells. When they were allowed to develop in suspension, YakA^{OE}/*gcsA*$^{-}$ cells formed aggregates which were similar to those of KAx3 cells regardless of the addition of GSH (Fig. 27). Interestingly, YakA^{OE}/KAx3 cells showed slightly faster rate of aggregation process compared to KAx3 cells (Fig. 28). YakA^{OE}/*gcsA*$^{-}$ cells developed in a comparable rate with KAx3 cells without GSH. These results confirm that GSH promotes induction of *yakA* expression to initiate development in *Dictyostelium*.

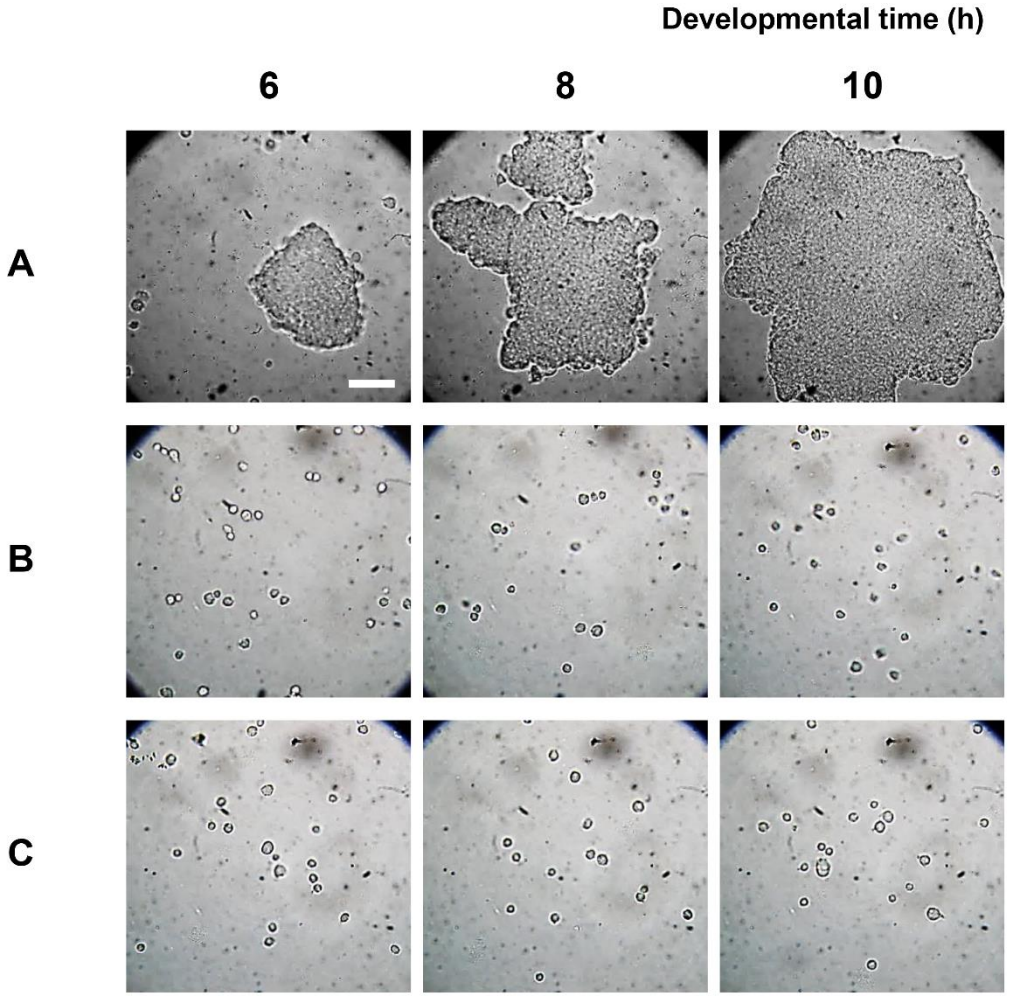


Fig. 26. Effect of GSH on development of *yakA*$^{-}$ cells in suspension. KAx3 and *yakA*$^{-}$ cells were subjected in non-nutrient KK2 buffer with nanomolar levels of cAMP pulses and 1 mM GSH. The progress of aggregation was observed for 10h and photographed at the indicated time. (A) KAx3 cells, (B) *yakA*$^{-}$ cells without GSH, (C) *yakA*$^{-}$ cells with 1 mM GSH. The scale bar represents 0.05 mm.

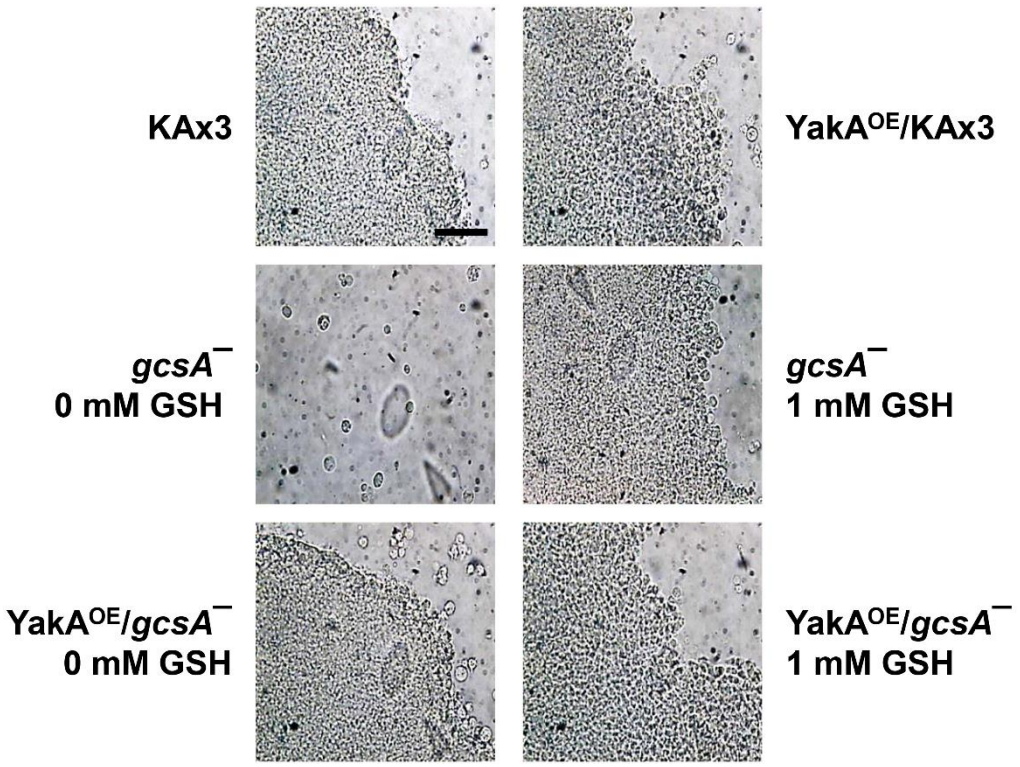


Fig. 27. Effect of YakA expression in *gcsA*$^{-}$ cells on developmental morphology. YakA-expressing KAx3 and *gcsA*$^{-}$ cells (YakA^{OE}/KAx3 and YakA^{OE}/*gcsA*$^{-}$ cells, respectively) were allowed to develop in non-nutrient KK2 buffer with exogenously added cAMP pulses and photographed at 12h. The scale bar represents 0.05mm.

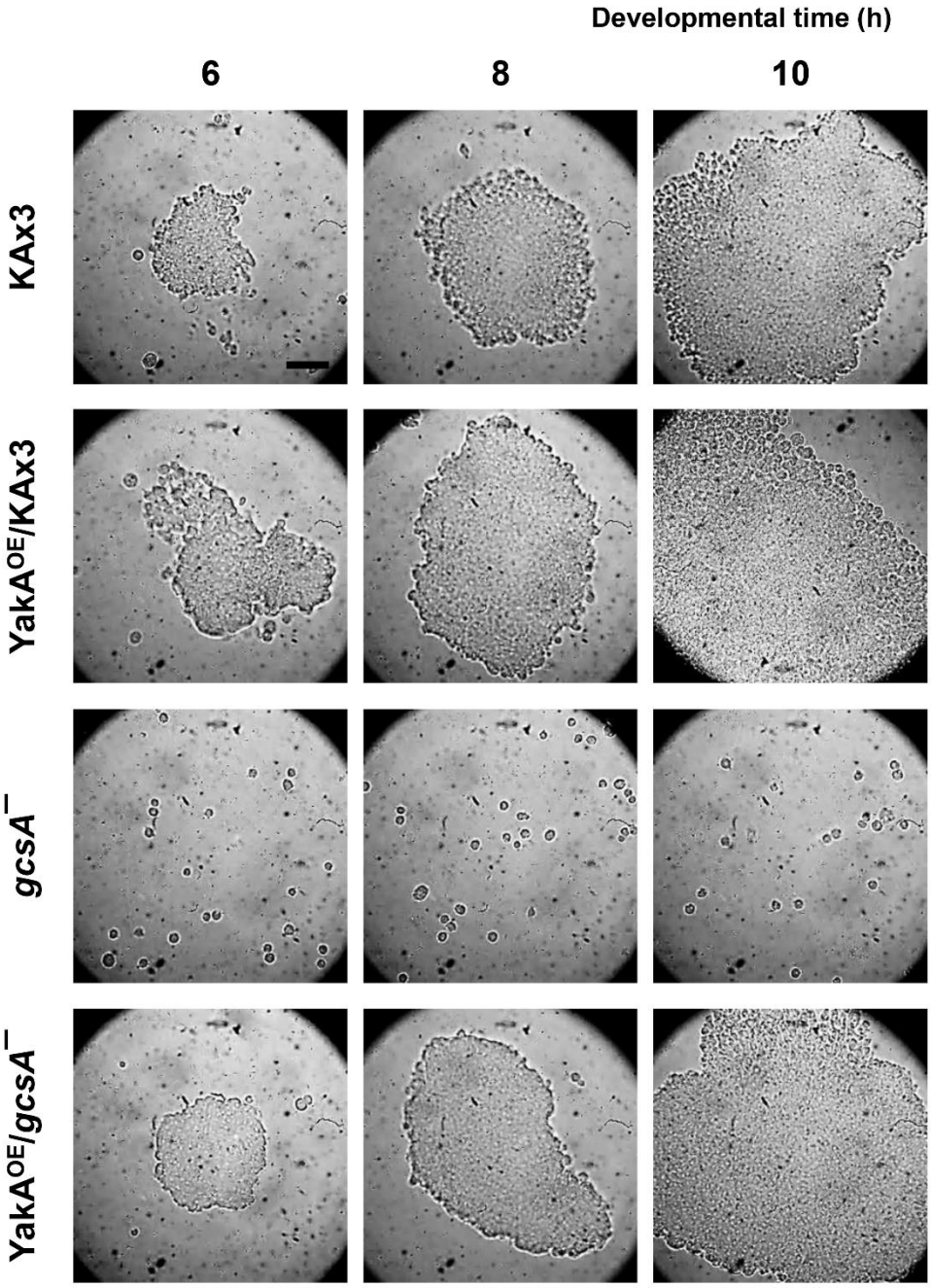


Fig. 28. Effect of YakA expression on the progress of aggregation. KAx3, YakA^{OE}/KAx3, *gcsA*$^{-}$, and YakA^{OE}/*gcsA*$^{-}$ cells were allowed to develop in non-nutrient KK2 buffer for 10 h with cAMP pulses and photographed at the indicated time. The scale bar represents 0.05 mm.

6.2. The effect of YakA expression on the expression of early developmental genes in *gcsA*$^{-}$ cells

gcsA^{-} cells developed without the addition of GSH by expressing YakA constitutively. Constitutive expression of YakA also influences the expression of early developmental genes in *gcsA*$^{-}$ cells. The expression of *carA*, *acaA*, and *pkaC* was significantly increased in *gcsA*$^{-}$ cells by the constitutive expression of YakA in *gcsA*$^{-}$ cells (YakA^{OE}/*gcsA*$^{-}$), although GSH was not added (Fig. 29). Further, the levels of *pufA* expression decreased in YakA^{OE}/*gcsA*$^{-}$ cells without the addition of GSH. Interestingly, the expression of *carA*, *acaA*, and *pkaC* also was significantly higher in YakA^{OE}/KAx3 cells than in *yakA*$^{-}$ cells (Fig. 29). These results demonstrate that the developmental defects in GSH-depleted *gcsA*$^{-}$ cells are due to the decreased expression of *yakA*. Thus, YakA expression rescues the expression of early developmental genes and eventually leads to proper development.

6.3. The effect of YakA expression on the concentration of intracellular GSH

To define the relation between YakA and GSH in *Dictyostelium*, intracellular concentration of GSH and total glutathione, which is sum of the GSH and GSSG levels, was analyzed during growth and development (Tables 4 and 5). To compare effectively the concentration of each cells, relative percentage values of reduced and total glutathione were calculated to the values of KAx3 cells (Figs 30 and 31). When cells were grown or starved in the absence of exogenously added GSH (*gcsA*$^{-}$ cells: 0 mM GSH; YakA^{OE}/*gcsA*$^{-}$ cells: 0 mM GSH), intracellular GSH and total glutathione were undetectable. When 1 mM GSH was added (*gcsA*$^{-}$ cells: 1 mM GSH;

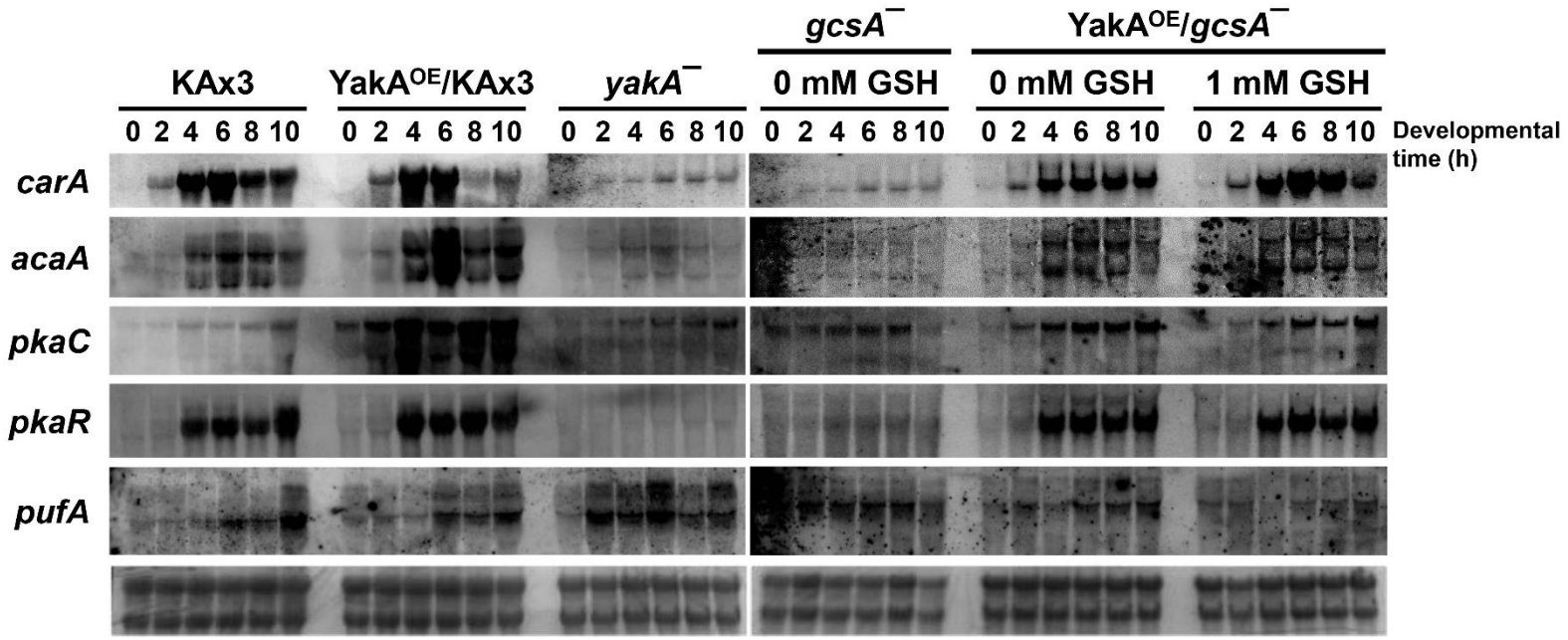


Fig. 29. Effect of YakA expression on early developmental gene expression. KAx3, YakA^{OE}/KAx3, *yakA*$^{-}$, *gcsA*$^{-}$, and YakA^{OE}/ *gcsA*$^{-}$ cells were allowed to develop in non-nutrient KK2 buffer and the expression of developmental genes was analyzed by Northern blotting. *carA*, encoding cAMP receptor 1; *acaA*, encoding adenylyl cyclase A; *pkaC*, protein kinase catalytic subunit; *pkaR*, encoding protein kinase regulatory subunit; *pufA*, encoding a RNA binding protein.

Table 4. Intracellular GSH contents of KAx3, *gcsA*$^{-}$, *yakA*$^{-}$, and YakA-expressing KAx3 and *gcsA*$^{-}$ cells[a] during growth

	GSH concentration (nmol/g[b])
KAx3	29.436 ± 2.993
YakA^{OE}/KAx3	33.606 ± 2.254
yakA^{-}	16.992 ± 2.757
gcsA^{-} 0 mM GSH	0.160 ± 0.461
gcsA^{-} 1 mM GSH	18.521 ± 2.287
YakA^{OE}/*gcsA*$^{-}$ 0 mM GSH	0.695 ± 1.389
YakA^{OE}/*gcsA*$^{-}$ 1 mM GSH	14.804 ± 2.072

[a] The values of quantitative measurements by HPLC represent mean ± S.E.M. of three independent experiments.

[b] **nmol/g**: wet weight

Table 5. Intracellular glutathione contents of KAx3, *gcsA*$^{-}$, *yakA*$^{-}$, and YakA-expressing KAx3 and *gcsA*$^{-}$ cells[a] during development

	Glutathione concentration nmol/g[b]		
	Reduced	Oxidized	Total
KAx3	26.591 ± 1.933	3.513 ± 2.668	30.105 ± 0.735
YakA^{OE}/KAx3	23.430 ± 2.148	8.602 ± 1.852	32.032 ± 0.295
yakA^{-}	10.745 ± 0.792	1.663 ± 1.915	12.408 ± 1.124
gcsA^{-} 0 mM GSH	0.418 ± 0.049	0.619 ± 0.084	1.037 ± 0.035
gcsA^{-} 1 mM GSH	2.505 ± 0.400	0.503 ± 0.865	3.007 ± 0.466
YakA^{OE}/*gcsA*$^{-}$ 0 mM GSH	0.660 ± 0.164	0.503 ± 0.120	1.163 ± 0.044
YakA^{OE}/*gcsA*$^{-}$ 1 mM GSH	2.312 ± 0.609	0.735 ± 0.936	3.047 ± 0.327

[a] The values of quantitative measurements by HPLC represent mean ± S.E.M. of three independent experiments.

[a] **nmol/g**: wet weight

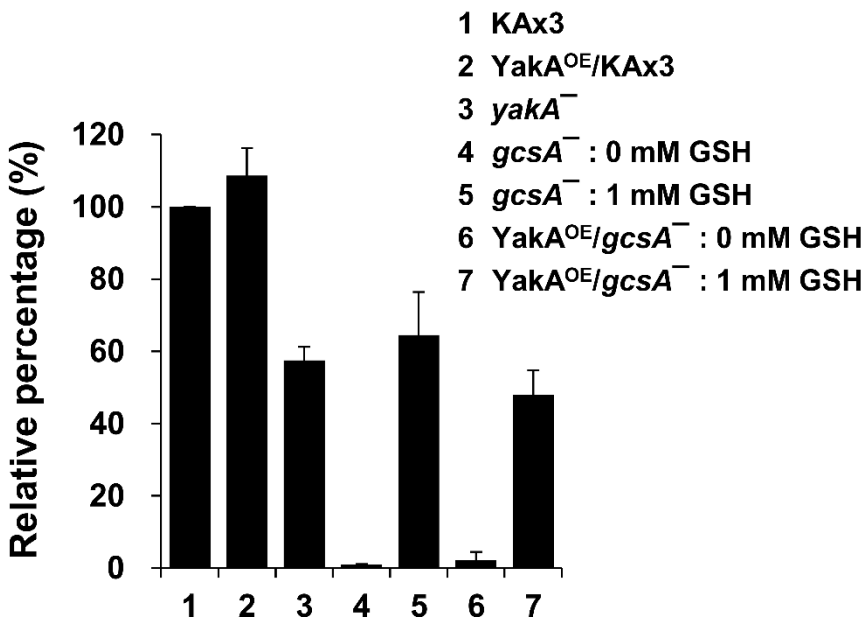
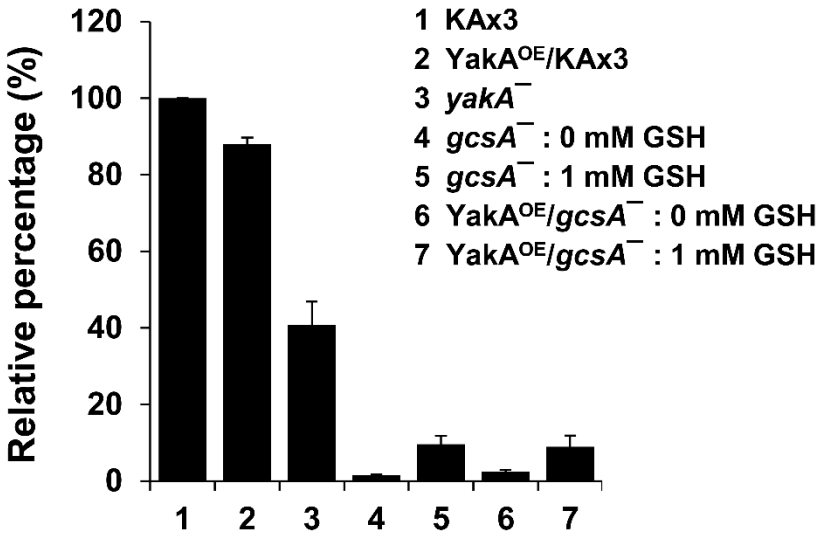


Fig. 30. Intracellular GSH contents of KAx3, *gcsA*¯, *yakA*¯, and YakA-expressing KAx3 and *gcsA*¯ cells during growth. The concentration of intracellular GSH was measured in exponentially growing KAx3, *gcsA*¯, *yakA*¯, and YakA-expressing KAx3 and *gcsA*¯ cells. The concentration was calculated in relative values compared to that of KAx3 cells. The values represent mean ± S.E.M. of three independent experiments.

A


Relative percentage (%)
120
100
80
60
40
20
0
1 2 3 4 5 6 7
1 KAx3
2 YakA^OE/KAx3
3 yakA¯
4 gcsA¯ : 0 mM GSH
5 gcsA¯ : 1 mM GSH
6 YakA^OE/gcsA¯ : 0 mM GSH
7 YakA^OE/gcsA¯ : 1 mM GSH

B

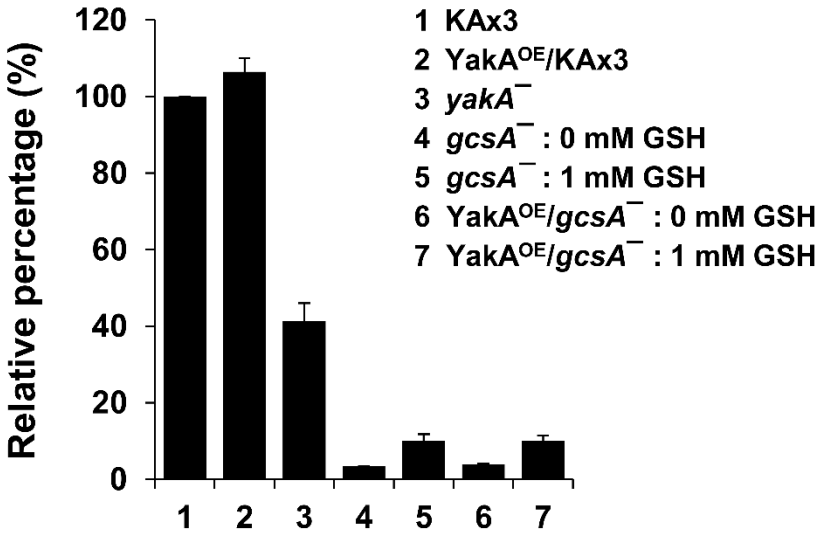

Relative percentage (%)
120
100
80
60
40
20
0
1 2 3 4 5 6 7
1 KAx3
2 YakA^OE/KAx3
3 yakA¯
4 gcsA¯ : 0 mM GSH
5 gcsA¯ : 1 mM GSH
6 YakA^OE/gcsA¯ : 0 mM GSH
7 YakA^OE/gcsA¯ : 1 mM GSH

Fig. 31. Intracellular glutathione contents of KAx3, *gcsA*$^{-}$, *yakA*$^{-}$, and YakA-expressing KAx3 and *gcsA*$^{-}$ cells during suspension development. The concentration of intracellular reduced and total glutathione was measured in KAx3, *gcsA*$^{-}$, *yakA*$^{-}$, and YakA-expressing KAx3 and *gcsA*$^{-}$ cells which were harvested at 0 h after development in suspension. The concentration of GSH (A) and the concentration of total glutathione (B), which is sum of the GSH and GSSG, were calculated in relative percentage values compared to that of KAx3 cells. The values represent mean ±S.E.M. of three independent experiments.

YakA^{OE}/*gcsA*$^{-}$ cells: 1 mM GSH) the intracellular concentration of GSH and total glutathione contents increased significantly during growth and development. And there were not significant differences between *gcsA*$^{-}$ and YakA^{OE}/*gcsA*$^{-}$ cells. In other words, the concentration of intracellular glutathione was not influenced by YakA expression in *gcsA*$^{-}$ cells. The concentration of GSH slightly increased during growth and decreased slightly during development, but the total glutathione level was higher in YakA^{OE}/KAx3 cells than that in KAx3 cells. The calculated concentration of GSSG was too low to detect and each cells showed similar levels of GSSG contents. It was interesting that YakA^{OE}/*gcsA*$^{-}$ cells proceeded to developmental life cycle although they contained intracellular GSH levels similar to those of *gcsA*$^{-}$ cells (See Discussion). These results suggest that intracellular GSH levels regulate the expression level of *yakA* but YakA does not effect on the concentration of intracellular GSH.

7. Relation between YakA and intracellular GSH

7.1. The intracellular contents of GSH in *yakA*$^{-}$ cells

To investigate the relation between GSH and YakA in detail, the concentration of intracellular GSH was measured in *yakA*$^{-}$ cells (Tables 3 and 4). In *yakA*$^{-}$ cells, the concentration of GSH and the total glutathione content were 40% and 60% lower in growing cells and in developing cells, respectively, than those in KAx3 cells (Figs 30 and 31). The results showed that intracellular GSH was significantly decreased in *yakA*$^{-}$ cells and slightly increased in YakA^{OE}/KAx3 cells. The concentration of intracellular GSH was affected by YakA expression in KAx3 cells (See Discussion).

7.2. The expression of *gcsA* in *yakA*$^{-}$ cells

Since the concentration of intracellular GSH seemed to be regulated by YakA in KAx3 cells, the expression levels of *gcsA* were examined in YakA^{OE}/KAx3 and *yakA*$^{-}$ cells. The expression of *gcsA* was difficult to detect in KAx3 cells at 0 h when cells were exposed to developmental conditions, gradually increased as cells formed aggregates, and reached a maximum at 10 h (Figs. 32 and 33). Interestingly, the level of *gcsA* mRNA was high in *yakA*$^{-}$ cells when development started (0 h). Further, the induced expression of *gcsA* mRNA was consistently maintained in *yakA*$^{-}$ cells throughout aggregation. The patterns of *gcsA* expression in KAx3 cells and YakA^{OE}/KAx3 cells were similar (Fig. 34). *yakA*$^{-}$ cells showed decreased intracellular GSH levels and increased *gcsA* expression. The levels of intracellular GSH and *gcsA* expression was not considerably changed in YakA^{OE}/*gcsA*$^{-}$ cells compared to those in KAx3 cells. These results indicate that the expression of *gcsA* is regulated transcriptionally in *yakA*$^{-}$ cells and this will be discussed more in Discussion section.

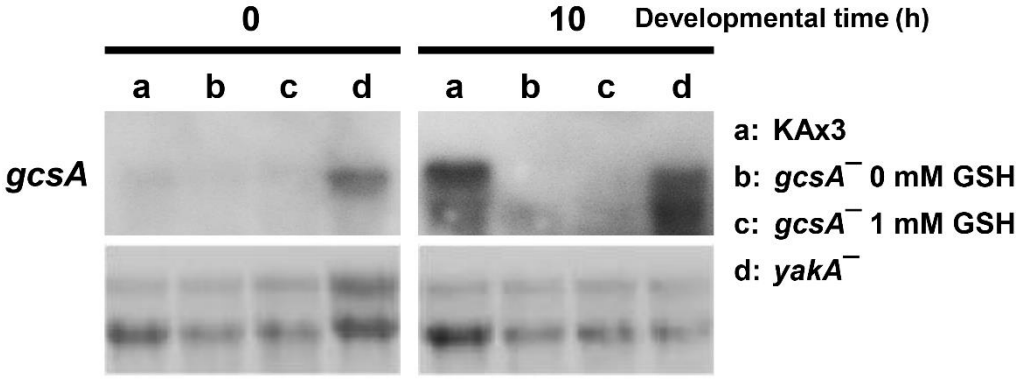


Fig. 32. Expression of *gcsA* in KAx3 and *yakA*¯ cells. KAx3, *yakA*, and *yakA*¯ cells were allowed to develop in non-nutrient KK2 buffer and total RNA was prepared at 0 h and at 10 h of development to analyze the expression *gcsA* by Northern blotting.

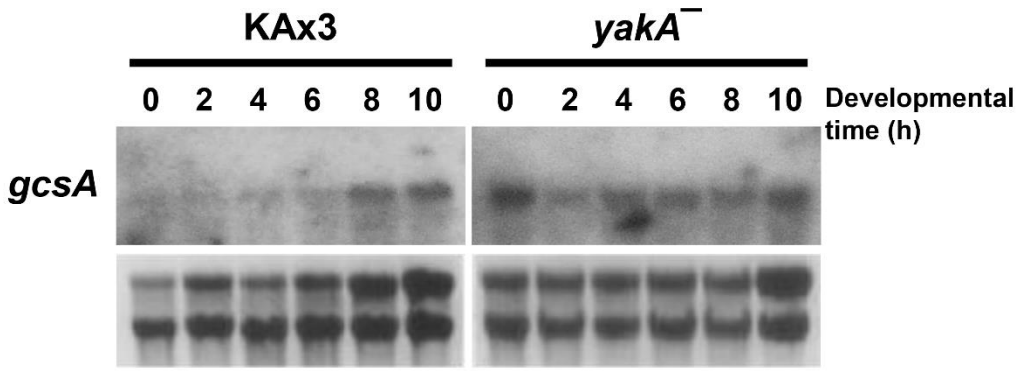


Fig. 33. Expression of *gcsA* in KAx3 and *yakA*$^{-}$ cells during aggregation progresses. KAx3 and *yakA*$^{-}$ cells were allowed to develop in non-nutrient KK2 buffer and total RNA was extracted at 2 h intervals. The expression of *gcsA* was monitored by Northern blotting analysis.

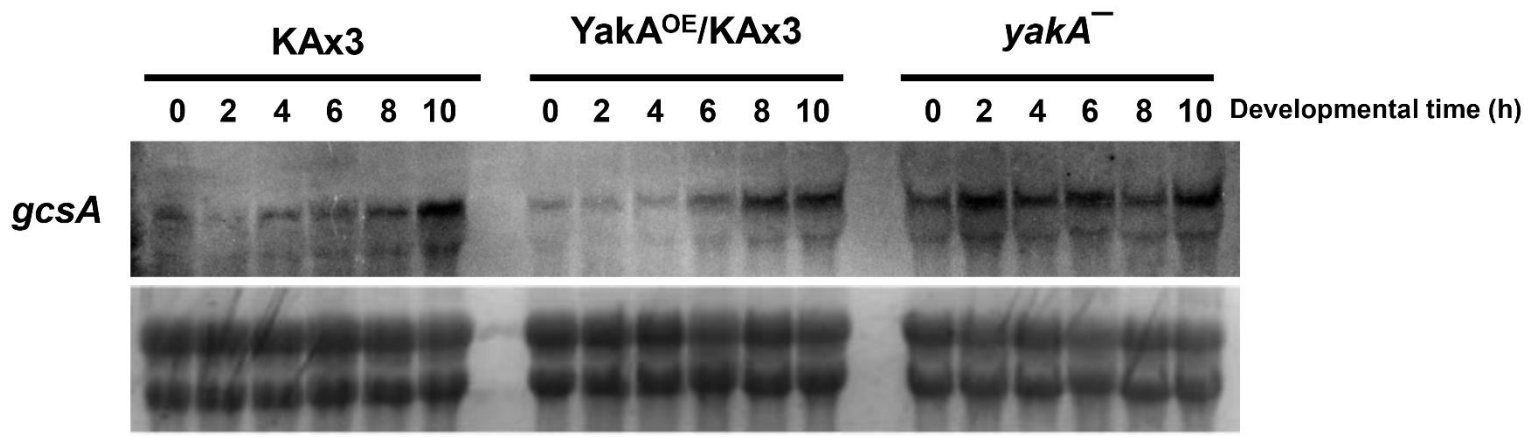


Fig. 34. Effect of YakA expression on the expression of *gcsA*. KAx3, YakA^{OE}/KAx3, and *yakA*$^{-}$ cells were allowed to develop in non-nutrient KK2 buffer and total RNA was extracted at 2 h intervals. The expression of *gcsA* was analyzed by Northern blotting.

IV. DISCUSSION

In the present study, the roles of GSH in *Dictyostelium* development were investigated by using GSH-depleted *gcsA*$^{-}$ cells defective in the synthesis of GSH. Previous reports have shown that GSH serves important roles in the normal growth and differentiation (Kim *et al.*, 2005; Choi *et al.*, 2006; Choi *et al.*, 2008). However the precise action mechanism responsible for developmental function of GSH is not unknown. According to findings in this study, intracellular GSH regulates the transition from growth to development by modulating the expression of *yakA* and its downstream regulators, which are essential for initiating development of *Dictyostelium*.

In previous research, *gcsA*$^{-}$ cells exhibit developmental defects according to the GSH concentration which is supplied in culture media before development. *gcsA*$^{-}$ cells are arrested at mound stage when pre-cultured with 0.2 mM GSH and at culmination step when pre-cultured with more than 0.5 mM GSH (Kim *et al.*, 2005). Prespore-specific genes and spore-specific genes are not expressed in *gcsA*$^{-}$ cells. In addition, the expression of *gcsA* is regulated during developmental life cycle of *Dictyostelium* (Kim *et al.*, 2005). The expression is increased during aggregation and during culmination, indicating that intracellular GSH has role in development of *Dictyostelium*. To understand the role of GSH, GSH was completely removed from *gcsA*$^{-}$ cells as described in material method. To minimize stresses of cells, the amount of added GSH was reduced gradually and incubated for 24 h in GSH-free media. As shown in Figs. 5 and 6, GSH-depleted *gcsA*$^{-}$ cells did not aggregate and also did not show chemotactical movements. *Dictyostelium* could not develop without GSH.

In general, GSH is known to be an essential metabolite and a major antioxidant in most eukaryotic cells. Disruption of GSH biosynthesis results in GSH auxotroph (Grant *et al.*, 1996; Chaudhuri *et al.*, 1997; Baek *et al.*, 2004)

and cell death through apoptosis (Hall, 1999; Madeo *et al.*, 1999; Baek *et al.*, 2004) in other organisms. In *Dictyostelium*, depletion of GSH by disrupting *gcsA* encoding GCS, the first enzyme in GSH biosynthesis, also caused GSH auxotroph but apoptosis was not induced. *gcsA*-null cells survived in rich media or in salt-only minimal media, though they showed cell cycle arrest or developmental defects. It was reasoned that the defects in development caused by GSH depletion may result from oxidative stress. Exogenous thiol compounds have been supplemented to compensate the role of GSH. NAC and DTT are cell permeable and stabilize the cellular reducing potential in other organisms (Abello *et al.*, 1994) and have protective role against oxidative stress (McGowan *et al.*, 1996; Reid *et al.*, 2005). DTT rescued the defects caused by oxidative stress by GSH depletion (Grant *et al.*, 1996). NAC also rescued apoptosis caused by GSH depletion (Shi *et al.*, 1994). It was found in this study that GSH, but not exogenous DTT and NAC rescued the developmental defect of the GSH-depleted *gcsA*$^{-}$ cells (Fig. 8). In addition, *gcsA*$^{-}$ cells did not develop with the addition of a general antioxidant, ascorbic acid. However, the oxidized form of GSH (GSSG) and the precursor of GSH (γ-GC), which can be converted into GSH, supported normal development of *gcsA*$^{-}$ cells, though it was not as much as GSH did. These results suggest that essential requirement of GSH in *Dictyostelium* development is probably not related to its redox properties. GSH itself has indispensable roles that cannot be compensated for by antioxidant.

The transition from growth to development is regulated by a complex series of signals designed to ensure that aggregation occurs under optimal conditions, especially through regulating gene expression. To understand the roles of GSH in development of *Dictyostelium*, the expression levels of several

genes which are required to initiate *Dictyostelium* development was determined. In absence of GSH, the expression of these genes was not precisely controlled in *gcsA*$^{-}$ cells. For example, *gcsA*$^{-}$ cells failed to decrease the expression of a vegetative-stage specific serine proteinase, *cprD* (Fig. 9), and the expression of *dscA* and *dia2* decreased in the GSH-depleted *gcsA*$^{-}$ cells (Fig. 10). The expression of *cprD*, *dscA*, and *dia2* act as markers for the transition from growth to development. *cprD* is expressed extensively during growth, but not during development. Members of the discoidin I gene family are among the first to be activated by prestarvation responses (Clarke *et al.*, 1987). During growth, cells secrete prestarvation factor (PSF) and estimate their density relative to the concentration of nutrients. When PSF reaches an appropriate concentration, it induces the expression of *dscA*, which encodes the discoidin I alpha chain, and prepares cells for developmental initiation. Discoidin accumulates continuously during early development until its transcription is inhibited by extracellular cAMP at the end of the aggregation phase. Thus, the expression of discoidin is an excellent indicator of the cell state in the developmental life cycle. *dia2* (differentiation-associated protein) transcripts accumulate exclusively in differentiating cells, but they are not detected in the growing cells (Chae *et al.*, 1998; Hirata *et al.*, 2008; Maeda, 2005). Interestingly, *dscA* and *dia2* were not expressed in GSH-depleted *gcsA*$^{-}$ cells (Fig. 10). However, exogenous addition of 1 mM GSH induced *dscA* and *dia2* expression. The expression of *cprD*, *dscA*, and *dia2* indicates that *Dictyostelium* cells did not initiate developmental cycle without GSH even in the presence of a developmental signal.

The early events in multicellular development of *Dictyostelium*, in particular the role of the cAMP signaling pathway, have been extensively studied (Loomis, 1998). cAMP signaling plays a central role in control of

multicellular aggregate formation. For the initiation of development, the expression of ACA and cAR1 is sophisticatedly regulated. The results analyzed in this study demonstrated that the expression of *carA* and *acaA* was not enhanced in the absence of GSH in *gcsA*$^{-}$ cells (Fig. 9). Failure of the induction of *carA* and *acaA* expression in *gcsA*$^{-}$ cells suggests a defect in the activation of cAMP-dependent signaling. Some groups reported that administering pulses of exogenous cAMP rescues the expression of *carA* and other components of cAMP signaling in some aggregation-defective mutants such as *gα3*$^{-}$ and Ddmyb2^{-} cells (Khosla *et al.*, 1996; Brandon and Podgorski, 1997; Otsuka and Van Haastert, 1998). However, pulsed addition of exogenous cAMP pulses did not rescue the expression of *carA* and *acaA* or developmental defect in *gcsA*$^{-}$ cells in the absence of GSH (Figs. 11 and 12). Therefore, it was suspected that g*csA*$^{-}$ cells might not response to exogenously added cAMP because of their lack of extracellular cAMP recognition. However, cAR1 expression also failed to produce aggregates. In the cAMP signaling system, the expression of ACA, cAR1, and Gα2 are regulated by positive feedback loop and enlarge cAMP pulses (Klein *et al.*, 1998; Pitt *et al.*, 1992; Kumagai *et al.*, 1989). Interestingly, the components of cAMP signaling pathway such as *carA*, *acaA*, and *gpaB* were expressed in sufficient amount to transmit cAMP signals in *gcsA*$^{-}$ cells in the absence of GSH (Fig. 15). These data demonstrate that lack of cAMP oscillations, the synthesis and recognition of cAMP, are not a main cause of the developmental defect in *gcsA*$^{-}$ cells. Thus, GSH may be required at a step upstream of cAMP signaling. Taken together, these findings strongly suggest that intracellular GSH plays essential roles in regulation of the transition from growth to development in *Dictyostelium*.

Next, the YakA signaling pathway was considered, which is known the earliest development regulating system prior to cAMP signaling in response to starvation signal. Previous reports have shown that YakA is necessary for the transition from growth to development in *Dictyostelium* (Souza *et al.*, 1998) and that the expression of YakA is required for the turning off growth-phase genes and for the induction of differentiation-associated genes. Moreover, *yakA*$^{-}$ cells show similar developmental phenotype and gene expression patterns to those of GSH-depleted *gcsA*$^{-}$ cells. *yakA*$^{-}$ cells and GSH-depleted *gcsA*$^{-}$ cells did not initiate development (Fig. 24) and showed undetectably low *carA* and *acaA* expression (Fig. 25). Surprisingly, the mRNA level of *yakA* significantly decreased in GSH-depleted *gcsA*$^{-}$ cells (Fig. 16). Further, the expression of *yakA* was modulated by the concentration of GSH added exogenously in KAx3 and *gcsA*$^{-}$ cells (Figs. 16 and 17). Therefore, these findings provide compelling evidence to support clearly the conclusion that GSH regulates the initiation of development by inducing the expression of *yakA*.

The role of GSH in regulating the expression of *yakA* was supported by the results acquired from monitoring other components of YakA pathway. As expected, similar to *yakA*$^{-}$ cells, GSH-depleted *gcsA*$^{-}$ cells showed increased *pufA* expression (Figs. 22 and 25) and decreased PKA activity (Fig. 23). Thus, it is apparent that the depletion of GSH blocks development because the YakA signaling system is not activated in the absence of GSH.

PKA activity was clearly regulated by the availability of intracellular GSH. The expression of *pkaC* and *pkaR* was also determined in *gcsA*$^{-}$ and *yakA*$^{-}$ cells. *pkaC* was transcribed normally as in KAx3 cells regardless of the presence of intracellular GSH or the expression of *yakA* (Figs. 22 and 25). In contrast, the expression of *pkaR* was unpredictable in *gcsA*$^{-}$ and *yakA*$^{-}$ cells. Increased

expression of *pkaR* was expected from the decreased PKA activity in GSH-depleted *gcsA*$^{-}$ cells and *yakA*$^{-}$ cells. However, the expression of *pkaR* was inhibited in both *gcsA*$^{-}$ and *yakA*$^{-}$ cells (Fig. 25) and further decreases was induced by the addition of GSH in both *gcsA*$^{-}$ and cAR1^{OE}/*gcsA*$^{-}$ cells (Figs. 15 and 22). YakA expression in KAx3 (YakA^{OE}/KAx3) and *gcsA*$^{-}$ (YakA^{OE}/*gcsA*$^{-}$) cells showed the increased expression of *pkaR* (Fig. 29). The analysis of Northern blotting showed that the transcriptional expression of *pkaR* was inhibited by GSH and induced by YakA. These findings suppose that the expression of *pkaR* is regulated in different way with the expression of *pkaC* and the transcriptional regulation of *pkaR* is not a critical factor to determine the activity of PKA.

To confirm the relationship between GSH and YakA, YakA was constitutively expressed in KAx3 and *gcsA*$^{-}$ cells (YakA^{OE}/KAx3 and YakA^{OE}/*gcsA*$^{-}$). GSH-depleted *gcsA*$^{-}$ cells developed and formed aggregates when YakA was expressed (Figs. 27 and 28). Moreover, the expression of developmental genes such as *carA*, *acaA*, and *pkaC* were increased by the constitutive expression of YakA in *gcsA*$^{-}$ cells (Fig. 29). These data imply that the developmental defects of GSH-depleted *gcsA*$^{-}$ cells are caused by the decreased expression of *yakA* and intracellular GSH induces the expression of *yakA* in response to a starvation signal to initiate developmental processes. Taken together, these findings suggest that intracellular GSH regulates the transition from growth to development by modulating YakA and downstream signaling.

The concentration of intracellular GSH in *yakA*$^{-}$ cells decreased by approximately 40% compared with KAx3 cells during growth and development (Figs. 30 and 31), although *gcsA* was constitutively expressed during growth

and aggregation processes (Figs. 32 and 33). In contrast, there were not significant differences of *gcsA* expression levels and intracellular GSH contents between in YakA^{OE}/KAx3 and KAx3 cells, indicating that YakA does not directly regulate intracellular GSH levels. According to Bloomfield and Pears (2003), a significant amount of superoxide is generated in response to CMF during the transition to the multicellular phase of development. Further, Taminato *et al.* (2002) reported that *yakA*$^{-}$ cells are hypersensitive to oxidative and nitrosoative stress. Therefore, it was postulated that the hypersensitive reaction to oxidative stress may cause the decrease in the intracellular GSH level in *yakA*$^{-}$ cells. Further, *yakA*$^{-}$ cells may consume more GSH than KAx3 cells to protect against oxidative stress and thus low concentration of intracellular GSH was detected. Increased *gcsA* expression could be explained by the decreased intracellular GSH level in *yakA*$^{-}$ cells. The decreased intracellular GSH level may induce *gcsA* expression through feedback regulation.

Furthermore, the intracellular content of GSH decreased by 90% in *gcsA*$^{-}$ cells which were developed with the addition of 1mM GSH compared to KAx3 cells when development commenced but at 10 h after development, the concentration rose to 40% of that in KAx3 cells (Fig. 7). In growing *gcsA*$^{-}$ cells, the GSH content was higher than that in developing cells but reached a limit of 50–60% of the level in KAx3 cells (Fig. 3), suggesting that exogenously added GSH is not completely incorporated by cells and that GSH enters *gcsA*$^{-}$ cells in proportion to the time of exposure. These results could explain the delayed developmental process and transcriptional expression of early developmental genes in *gcsA*$^{-}$ cells which were developed with 1 mM GSH. Exogenously added GSH did not cross the plasma membrane completely and decreased intracellular GSH compare to KAx3 cells result in incomplete restoration of

developmental defect. Remarkably, a very low level of GSH can induce the development of *gcsA*$^{-}$ cells, suggesting that intracellular GSH plays a vital role in *Dictyostelium* development.

Finally, a model of developmental initiation in which GSH regulates the expression of YakA and other components of the YakA signaling pathway is proposed (Fig. 35). GSH plays an essential role in the transition from growth to development by regulating YakA and signal transduction when development initiates. It is expected that further studies designed to elucidate the molecular mechanisms that govern the regulation of gene transcription by GSH may provide insight into general mechanisms underlying initiation of cell development.

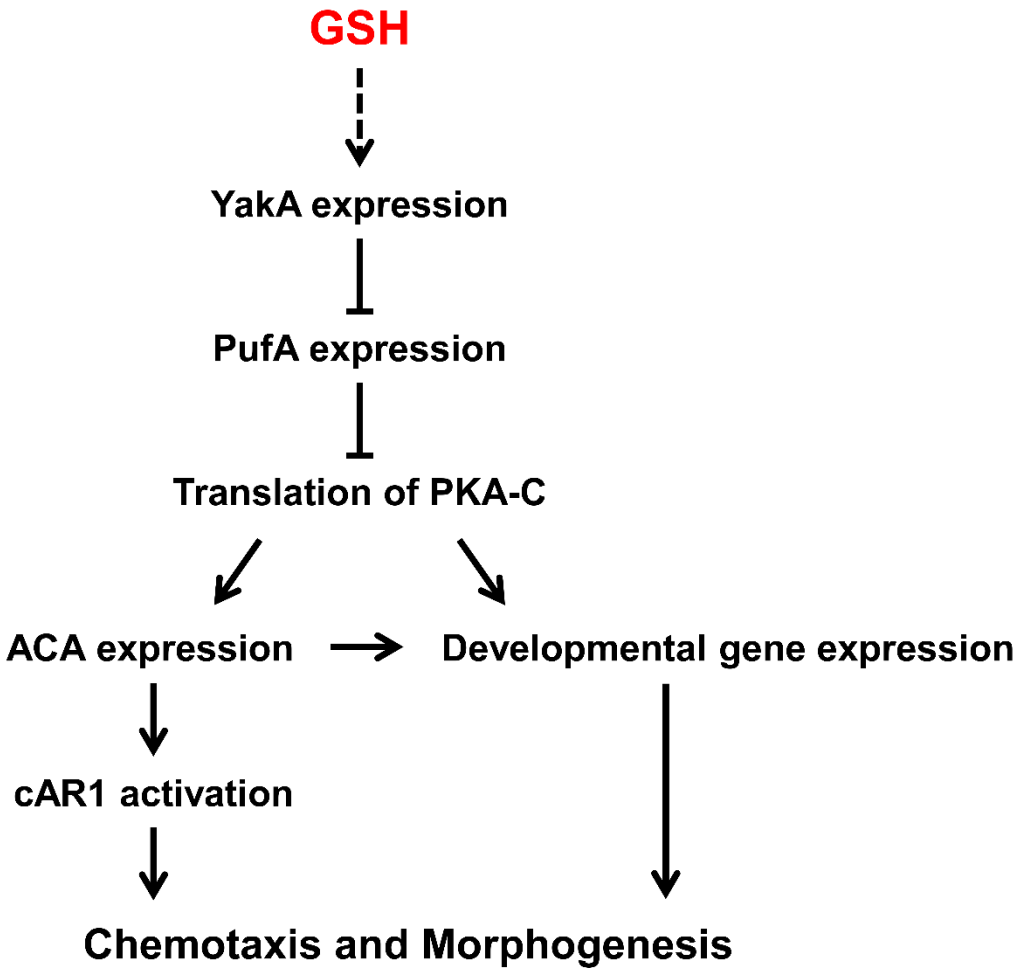


Fig. 35. Regulation of developmental initiation by intracellular GSH in *Dictyostelium discoideum*.

V. REFERENCES

Abe, K., and Yanagisawa, K. (1983) A new class of rapidly developing mutants in *Dictyostelium discoideum*: implications for cyclic AMP metabolism and cell differentiation. *Dev. Biol.* **95**, 200-210.

Abello, P.A., Fidler, S.A., and Buchman, T.G. (1994) Thiol reducing agents modulate induced apoptosis in porcine endothelial cells. *Shock* **2**, 79-83.

Akerboom, T. P., Bilzer, M., and Sies, H. (1982) The relationship of biliary glutathione disulfide efflux and intracellular glutathione disulfide content in perfused rat liver. *J. Biol. Chem.* **257**, 4248-4252.

Allen, R. G., Newton, R. K., Sohal, R. S., Shipley, G. L., and Nations, C. (1985) Alterations in superoxide dismutase, glutathione, and peroxides in the plasmodial slime mold *Physarum polycephalum* during differentiation. *J. Cell Physiol.* **125**, 413-419.

Anjard, C., Etchebehere, L., Pinaud, S., Veron, M., and Reymond, C. D. (1993) An unusual catalytic subunit for the cAMP-dependent protein kinase of *Dictyostelium discoideum*. *Biochemistry* **32**, 9532-9538.

Anjard, C., Pinaud, S., Kay, R. R., and Reymond, C. D. (1992) Overexpression of Dd PK2 protein kinase causes rapid development and affects the intracellular cAMP pathway of *Dictyostelium discoideum*. *Development* **115**, 785-790.

Aw, T. Y. (2003) Cellular Redox: A modulator of intestinal epithelial cell proliferation. *Physiology* **18**, 201-204.

Baek, Y. U., Kim, Y. R., Yim, H. S., and Kang, S. O. (2004) Disruption of gammaglutamylcysteine synthetase results in absolute glutathione auxotrophy and apoptosis in *Candida albicans*. *FEBS Lett.* **556**, 47-52.

Bella, D. L., Hirschberger, L. L., Hosokawa, Y., and Stipanuk, M. H. (1999) Mechanisms involved in the regulation of key enzymes of cysteine metabolism in rat liver *in vivo*. *Am. J. Physiol.* **276**, E326-335.

Bloomfield, G., and Pears, C. (2003) Superoxide signalling required for multicellular development of *Dictyostelium*. *J. Cell Sci.* **16**, 3387-3397.

Brandon, M. A., and Podgorski, G. J. (1997) G alpha 3 regulates the cAMP signaling system in *Dictyostelium*. *Mol. Biol. Cell* **8**, 1677-1685.

Cairns, N. G., Pasternak, M., Wachter, A., Cobbett, C. S., and Meyer, A. J. (2006) Maturation of *Arabidopsis* seeds is dependent on glutathione biosynthesis within the embryo. *Plant Physiol.* **141**, 446-455.

Chae, S. C., Inazu, Y., Amagai, A., and Maeda, Y. (1998) Underexpression of a novel gene, *dia2*, impairs the transition of *Dictyostelium* cells from growth to differentiation. *Biochem. Biophys. Res. Commun.* **252**, 278-283.

Chaudhuri, B., Ingavale, S., and Bachhawat, A. K. (1997) *apd1+*, a gene required for red pigment formation in *ade6* mutants of *Schizosaccharomyces pombe*, encodes an enzyme required for glutathione biosynthesis: a role for glutathione and a glutathione-conjugate pump. *Genetics* **145**, 75-83.

Chen, M. Y., Long, Y., and Devreotes, P. N. (1997) A novel cytosolic regulator, Pianissimo, is required for chemoattractant receptor and G protein-mediated activation of the 12 transmembrane domain adenylyl cyclase in *Dictyostelium*. *Genes Dev.* **11**, 3218-3231.

Choi, C. H., Kim, B. J., Jeong, S. Y., Lee, C. H., Kim, J. S., Park, S. J., Yim, H. S. and Kang, S. O. (2006) Reduced glutathione levels affect the culmination and cell fate decision in *Dictyostelium discoideum*. *Dev. Biol.* **295**, 523-533.

Choi, C. H., Park, S. J., Jeong, S. Y., Yim, H. S., and Kang, S. O. (2008) Methylglyoxal accumulation by glutathione depletion leads to cell cycle arrest in *Dictyostelium*. *Mol. Microbiol.* **70**, 1293-1304.

Clarke, M., Kayman, S. C., and Riley, K. (1987) Density-dependent induction of discoidin-I synthesis in exponentially growing cells of *Dictyostelium discoideum*. *Differentiation* **34**, 79-87.

Clarke, M., Yang, J., and Kayman, S. C. (1988) Analysis of the prestarvation response in growing cells of *Dictyostelium discoideum*. *Dev. Genet.* **9**, 315-326.

Cocucci, S.M. and Sussman, M. (1970) RNA in cytoplasmic and nuclear fractions of cellular slime mold amebas. *J. Cell Sci.* **45**, 399-407.

Cotter, D. A., Dunbar, A. J., Buconjic, S. D., and Wheldrake, J. F. (1999) Ammonium phosphate in sori of *Dictyostelium discoideum* promotes spore dormancy through stimulation of the osmosensor ACG. *Microbiology* **145**, 1891-1901.

de Gunzburg, J., Franke, J., Kessin, R. H., and Veron, M. (1986) Detection and developmental regulation of the mRNA for the regulatory subunit of the cAMP-dependent protein kinase of *D. discoideum* by cell-free translation. *EMBO J.* **5**, 363-367.

Devreotes, P. N. (1983) Cyclic nucleotides and cell-cell communication in Dictyostelium discoideum. Advances in cyclic nucleotide research.

Devreotes, P.N. (1994) G protein-linked signaling pathways control the developmental program of *Dictyostelium*. *Neuron* **12**, 235-241.

Devreotes, P. N., and Zigmond, S. H. (1988) Chemotaxis in eukaryotic cells: a focus on leukocytes and *Dictyostelium*. *Annu. Rev. Cell Biol.* **4**, 649-686.

Dinauer, M. C., Mackay, S. A., and Devreotes, P. N. (1980) Cyclic 3', 5'-AMP relay in *Dictyostelium discoideum* III. The relationship of cAMP synthesis and secretion during the cAMP signaling response. *J. Cell Biol.* **86**, 537-544.

Dumollard, R., Ward, Z., Carroll, J., and Duchen, M. R. (2007) Regulation of redox metabolism in the mouse oocyte and embryo. *Development* **134**, 455-465.

Durston, A. J. (1976) Tip formation is regulated by an inhibitory gradient in the *Dictyostelium discoideum* slug. *Nature* London: **263**, 126-129.

Eichinger, L., Pachebat, J. A., Glöckner, G., Rajandream, M. A., Sucgang, R., Berriman, M., Song, J., Olsen, R., Szafranski, K., Xu, Q., Tunggal, B.,

Kummerfeld, S., Madera, M., Konfortov, B. A., Rivero, F., Bankier, A. T., Lehmann, R., Hamlin, N., Davies, R., Gaudet, P., Fey, P., Pilcher, K., Chen, G., Saunders, D., Sodergren, E., Davis, P., Kerhornou, A., Nie, X., Hall, N., Anjard, C., Hemphill, L., Bason, N., Farbrother, P., Desany, B., Just, E., Morio, T., Rost, R., Churcher, C., Cooper, J., Haydock, S., van Driessche, N., Cronin, A., Goodhead, I., Muzny, D., Mourier, T., Pain, A., Lu, M., Harper, D., Lindsay, R., Hauser, H., James, K., Quiles, M., Madan Babu, M., Saito, T., Buchrieser, C., Wardroper, A., Felder, M., Thangavelu, M., Johnson, D., Knights, A., Loulseged, H., Mungall, K., Oliver, K., Price, C., Quail, M. A., Urushihara, H., Hernandez, J., Rabbinowitsch, E., Steffen, D., Sanders, M., Ma, J., Kohara, Y., Sharp, S., Simmonds, M., Spiegler, S., Tivey, A., Sugano, S., White, B., Walker, D., Woodward, J., Winckler, T., Tanaka, Y., Shaulsky, G., Schleicher, M., Weinstock, G., Rosenthal, A., Cox, E. C., Chisholm, R. L., Gibbs, R., Loomis, W. F., Platzer, M., Kay, R. R., Williams, J., Dear, P. H., Noegel, A. A., Barrell, B., and Kuspa, A. (2005) The genome of the social amoeba *Dictyostelium discoideum*. *Nature* **435**, 43-57.

Fang, Y. Z., Yang, S., and Wu, G. (2002) Free radicals, antioxidants, and nutrition. *Nutrition* **18**, 872-879.

Feinberg, A. P., and Vogelstein, B. (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132**, 6-13.

Firtel, R. A. (1995) Integration of signaling information in controlling cell fate decisions in *Dictyostelium. Genes Dev.* **9**, 1427-1444.

Forbes, A., and Lehmann, R. (1998) Nanos and Pumilio have critical roles in the development and function of *Drosophila* germline stem cells. *Development* **125**, 679-690.

Franklin, C. C., Rosenfeld-Franklin, M. E., White, C., Kavanagh, T. J., and Fausto, N. (2003) TGFβ1-induced suppression of glutathione antioxidant defenses in hepatocytes: caspase-dependent post-translational and caspase-independent transcriptional regulatory mechanisms. *FASEB J.* **17**, 1535-1537.

Galardi-Castilla, M., Pergolizzi, B., Bloomfield, G., Skelton, J., Ivens, A., Kay, R. R., Bozzaro, S., and Sastre, L. (2008) SrfB, a member of the Serum Response Factor family of transcription factors, regulates starvation response and early development in *Dictyostelium*. *Dev. Biol.* **316**, 260-274.

Galter, D., Mihm, S., and Droge, W. (1994) Distinct effects of glutathione disulphide on the nuclear transcription factor kappa B and the activator protein-1. *Eur. J. Biochem.* **221**, 639-648.

Garcia-Ruiz, C., and Fernandez-Checa, J. C. (2006) Mitochondrial glutathione: hepatocellular survival-death switch. *J. Gastroenterol. Hepatol.* (*Suppl*) **21**, S3-S6.

Garcia-Ruiz, C., and Fernández-Checa, J. C. (2007) Redox regulation of hepatocyte apoptosis. *J. Gastroenterol. Hepatol.* (*Suppl*) **22**, S38-S42.

Gardiner, C. S., and Reed, D. J. (1994) Status of glutathione during oxidant-induced oxidative stress in the preimplantation mouse embryo. *Biol.Reprod.* **51**, 1307-1314.

Gomer, R. H., Yuen, I. S., and Firtel, R. A. (1991) A secreted 80 x 10(3) Mr protein mediates sensing of cell density and the onset of development in *Dictyostelium***.** *Development* **112**, 269-278.

Gomi, A., Masuzawa, T., Ishikawa, T., and Kuo, M. T. (1997) Posttranscriptional regulation of MRP/GS-X pump and γ-glutamylcysteine synthetase expression by 1-(4-amino-2-methyl-5-pyrimidinyl)-methyl-3-(2-chloroethyl)-3-nitrosourea and by cycloheximide in human glioma cells. *Biochem. Biophys. Res. Commun.* **239**, 51-56.

Grant, C. M., MacIver, F. H., and Dawes, I. W. (1996) Glutathione is an essential metabolite required for resistance to oxidative stress in the yeast *Saccharomyces cerevisiae*. *Curr. Genet.* **29**, 511–515.

Grant, C. M., MacIver, F. H., and Dawes, I. W. (1997) Glutathione synthetase is dispensable for growth under both normal and oxidative stress conditions in the yeast *Saccharomyces cerevisiae* due to an accumulation of the dipeptide gamma-glutamylcysteine. *Mol. Biol. Cell* **8**, 1699-1707.

Griffith, O. W., and Mulcahy, R. T. (1999) The enzymes of glutathione synthesis: γ-glutamylcysteine synthetase. *Adv. Enzymol. Relat. Areas Mol. Biol.* **73**, 209-267.

Grinberg, L., Fibach, E., Amer, J., and Atlas, D. (2005) *N*-acetylcysteine amide, a novel cell-permeating thiol, restores cellular glutathione and protects human red blood cells from oxidative stress, *Free Radical Biol. Med.* **38**, 136-145.

Gross, J. D. (1994) Developmental decisions in *Dictyostelium discoideum*. *Microbiol. Rev.* **58**, 330-351.

Hall, A. G. (1999) The role of glutathione in the regulation of apoptosis. *Eur. J. Clin. Invest.* **29**, 238-245.

Halliwell, B., and Gutteridge, J. M. C. (1989) Free Radicals in Biology and Medicine. London: Oxford Univ. Press.

Harwood, A. J., Hopper, N. A., Simon, M. N., Bouzid, S., Veron, M., and Williams, J. G. (1992) Multiple roles for cAMP-dependent protein kinase during *Dictyostelium* development. Dev. Biol. 149, 90-99.

Hayes, J. D., and McLellan, L. I. (1999) Glutathione and glutathione-dependent enzymes represent a co-ordinately regulated defence against oxidative stress. *Free Radic. Res.* **31**, 273-300.

Hirata, K., Amagai, A., Chae, S. C., Hirose, S., and Maeda, Y. (2008) Involvements of a novel protein, DIA2, in cAMP signaling and spore differentiation during *Dictyostelium* development. *Differentiation* **76**, 310-322.

Hopper, N. A., Anjard, C., Reymond, C. D., and Williams, J. G. (1993a) Induction of terminal differentiation of *Dictyostelium* by cAMP-dependent protein kinase and opposing effects of intracellular and extracellular cAMP on stalk cell differentiation. *Development* 119, 147-154.

Hwang, C., Sinskey, A. J., and Lodish, H. F. (1992) Oxidized redox state of glutathione in the endoplasmic reticulum. *Science* **257**, 1496-1502.

Iijima, N., Takagi, T., and Maeda, Y. (1995) A Proteinous Factor Mediating Intercellular Communication during the Transition of *Dictyostelium* Cells from Growth to Differentiation. *Zool. Sci.* **12**, 61-69.

Insall, R., Kuspa, A., Lilly, P. J., Shaulsky, G., Levin, L. R., Loomis, W. F., and Devreotes, P. (1994) CRAC, a cytosolic protein containing a pleckstrin homology domain, is required for receptor and G protein-mediated activation of adenylyl cyclase in *Dictyostelium*. *J. Cell Biol.* **126**, 1537-1545.

Jacquet, M., Guilbaud, R., and Garreau, H. (1988) Sequence analysis of the DdPYR5-6 gene coding for UMP synthase in *Dictyostelium discoideum* and comparison with orotate phosphoribosyl transferases and OMP decarboxylases. *Mol. Gen. Genet.* **211**, 441-445.

Johnson, R. L., Saxe, C. 3rd., Gollop, R., Kimmel, A. R., and Devreotes, P. N. (1993) Identification and targeted gene disruption of cAR3, a cAMP receptor subtype expressed during multicellular stages of *Dictyostelium* development. *Genes Dev.* **7**, 273-282.

Johnson, R. L., Vaughan, R. A., Caterina, M. J., Van Haastert, P. J., and Devreotes, P. N. (1991) Overexpression of the cAMP receptor 1 in growing *Dictyostelium* cells. *Biochemistry* **30**, 6982-6986.

Kaplowitz, N., Aw, T. Y., and Ookhtens, M. (1985) The regulation of hepatic glutathione. *Annu. Rev. Pharmacol. Toxicol.* **25**, 715-744.

Katoh, M., Chen, G., Roberge, E., Shaulsky, G., and Kuspa, A. (2007) Developmental commitment in *Dictyostelium discoideum*. *Eukaryotic cell* **6**, 2038-2045.

Kawamura, N. (1960) Cytochemical and quantitative study of protein-bound sulfhydryl and disulfide groups in eggs of *Arbacia* during the first cleavage. *Exp. Cell Res.* **20**, 127-138.

Ken, R., and Singleton, C. K. (1994) Redundant regulatory elements account for the developmental control of a ribosomal protein gene of *Dictyostelium discoideum*. *Differentiation* **55**, 97-103.

Ketterer, B., Coles, B., and Meyer, D. J. (1983) The role of glutathione in detoxication. *Environ. Health Perspect.* **49**, 59-69.

Kesbeke, F, Snaar-Jagalska, B. E., and Van Haastert, P. J. (1988) Signal transduction in *Dictyostelium fgd* A mutants with a defective interaction between surface cAMP receptors and a GTP-binding regulatory protein. *J. Cell Biol.* **107**, 521-528.

Khosla, M., Spiegelman, G. B., and Weeks, G. (1996) Overexpression of an activated *rasG* gene during growth blocks the initiation of *Dictyostelium* development. *Mol. Cell Biol.* **16**, 4156-4162.

Kim, B. J., Choi, C. H., Lee, C. H., Jeong, S. Y., Kim, J. S., Kim, B. Y., Yim, H. S. and Kang, S. O. (2005) Glutathione is required for growth and prespore cell differentiation in *Dictyostelium*. *Dev. Biol.* **284**, 387-398.

Kim, J. S., Seo, J. H., Yim, H. S., and Kang, S. O. (2011) Homeoprotein Hbx4 represses the expression of the adhesion molecule DdCAD-1 governing cytokinesis and development. *FEBS Lett.* **585**, 1864-1872.

Klein, P. S., Sun, T. J., Saxe, C. L. 3rd., Kimmel, A. R., Johnson, R. L., and Devreotes, P. N. (1988) A chemoattractant receptor controls development in *Dictyostelium discoideum*. *Science* **241**, 1467-1472.

Klein, P., Theibert, A., and Devreotes, P. (1988) Identification and ligand-induced modification of the cAMP receptor in *Dictyostelium*. *Methods Enzymol.* **159**, 267-278.

Kriebel, P. W., Barr, V. A., and Parent, C. A. (2003) Adenylyl cyclase localization regulates streaming during chemotaxis. *Cell* **112**, 549-560.

Kumagai, A., Pupillo, M., Gundersen, R., Miake-Lye, R., Devreotes, P. N., and Firtel, R. A. (1989) Regulation and function of G alpha protein subunits in *Dictyostelium*. *Cell* **57**, 265-275.

Lacombe, M. L., Podgorski, G. J., Franke, J., and Kessin, R. H. (1986) Molecular cloning and developmental expression of the cyclic nucleotide phosphodiesterase gene of *Dictyostelium discoideum*. *J. Biol. Chem.* **261**, 16811-16817.

Levi, S., Polyakov, M., and Egelhoff, T. T. (2000) Green fluorescent protein and epitope tag fusion vectors for *Dictyostelium discoideum*. *Plasmid* **44**, 231-238.

Lewin, S. (1976) Vitamin C: Its molecular biology and medical potential. New York, NY: Academic press 42-59.

Lilly, P., Wu, L. I. J. U. N., Welker, D. L., and Devreotes, P. N. (1993) A G-protein beta-subunit is essential for *Dictyostelium development*. *Genes Dev.* **7**, 986-995.

Lilly, P. J., and Devreotes, P. N. (1994) Identification of CRAC, a cytosolic regulator required for guanine nucleotide stimulation of adenylyl cyclase in *Dictyostelium*. *J. Biol. Chem.* **269**, 14123-14129.

Lilly, P. J., and Devreotes, P. N. (1995) Chemoattractant and GTP gamma S-mediated stimulation of adenylyl cyclase in *Dictyostelium* requires translocation of CRAC to membranes. *J. Cell Biol.* **129**, 1659-1665.

Lindermayr, C., Sell, S., Müller, B., Leister, D., and Durner, J. (2010) Redox regulation of the NPR1-TGA1 system of Arabidopsis thaliana by nitric oxide. *Plant Cell* **22**, 2894-2907.

Loomis, W. F. (1982) The spatial pattern of cell-type differentiation in *Dictyostelium*. *Dev. Biol.* **93**, 279-284.

Loomis, W. F. (1998) Role of PKA in the timing of developmental events in *Dictyostelium* cells. *Microbiol. Mol. Biol. Rev.* **62**, 684-694.

Louis, J. M., Ginsburg, G. T., and Kimmel, A. R. (1994) The cAMP receptor CAR4 regulates axial patterning and cellular differentiation during late development of *Dictyostelium*. *Genes Dev.* **8**, 2086-2096.

Louis, J. M., Saxe, C. L. 3rd., and Kimmel, A. R. (1993) Two transmembrane signaling mechanisms control expression of the cAMP receptor gene CAR1 during *Dictyostelium* development. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 5969-5973.

Lu, S. C. (1999) Regulation of hepatic glutathione synthesis: current concepts and controversies. *FASEB J.* **13**, 1169-1183.

Lu, S. C. (2000) Regulation of glutathione synthesis. *Curr. Top. Cell Regul.* **36**, 95-116.

Lu, S. C. (2009) Regulation of glutathione synthesis. *Mol. Aspects Med.* **30**, 42-59.

Luberda, Z. (2005) The role of glutathione in mammalian gametes. *Reprod. Biol.* **5**, 5-17.

McGowan, A.J., Fernandes, R.S., Samali, A., and Cotter, T.G. (1996) Anti-oxidants and apoptosis. *Biochem. Soc. Trans.* **24**, 229-232.

MacWilliams, H. K. (1982) Transplantation experiments and pattern mutants in the *Dictyostelium discoideum* slug. *Symp. Soc. Dev. Biol.* **40**, 463-483.

Madeo, F., Frohlich, E., Ligr, M., Grey, M., Sigrist, S. J., Wolf, D. H., and Frohlich, K. U. (1999) Oxygen stress: a regulator of apoptosis in yeast. *J. Cell Biol.* **145**, 757-767.

Mahadeo, D. C., and Parent, C. A. (2006) Signal Relay During the Life Cycle of *Dictyostelium*. *Curr. Top. Dev. Biol.* **73**, 115-140.

Maeda, Y. (2005) Regulation of growth and differentiation in *Dictyostelium*. *Int. Rev. Cytol.* **244**, 287-332.

Maeda, M., Aubry, L., Insall, R., Gaskins, C., Devreotes, P. N., and Firtel, R. A. (1996) Seven helix chemoattractant receptors transiently stimulate mitogen-activated

protein kinase in *Dictyostelium*. Role of heterotrimeric G proteins. *J. Biol. Chem.* **271**, 3351-3354.

Maeda, Y., and Iijima, N. (1992) Cross-talks required for the acquisition of development competence in *Dictyostelium discoideum* cells. *Anim. Biol.* **1**, 145-155.

Mann, S. K., and Firtel, R. A. (1989) Two-phase regulatory pathway controls cAMP receptor-mediated expression of early genes in *Dictyostelium*. *Proc. Natl. Acad. Sci. U.S.A.* **86**, 1924-1928.

Mann, S. K., and Firtel, R. A. (1991) A developmentally regulated, putative serine/threonine protein kinase is essential for development in *Dictyostelium*. *Mech. Dev.* **35**, 89-101.

Mann, S. K., Yonemoto, W. M., Taylor, S. S., and Firtel, R. A. (1992) DdPK3, which plays essential roles during *Dictyostelium* development, encodes the catalytic subunit of cAMP-dependent protein kinase. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 10701-10705.

Mann, S. K., Brown, J. M., Briscoe, C., Parent, C., Pitt, G., Devreotes, P. N., and Firtel, R. A. (1997) Role of cAMP-dependent protein kinase in controlling aggregation and postaggregative development in *Dictyostelium*. *Dev. Biol.* **183**, 208-221.

Margolskee, J. P., and Lodish, H. F. (1980) The regulation of the synthesis of actin and two other proteins induced early in *Dictyostelium discoideum* development. *Dev. Biol.* **74**, 50-64.

McPherson, C. E., and Singleton, C. K. (1992) V4, a gene required for the transition from growth to development in *Dictyostelium discoideum*. *Dev. Biol.* **150**, 231-242.

Meister, A., and Anderson, M. E. (1983) Glutathione. *Annu. Rev. Biochem.* **52**, 711-760.

Meister, A. (1988) Glutathione. The Liver: Biology and Pathobiology, second Ed. New York: Raven Press 401-417.

Meister, A. (1994) Glutathione, ascorbate, and cellular protection. *Cancer Res. (Suppl)* **54**, 1969s-1975s.

Milne J. L., and Coukell, M. B. (1991) A Ca2+ transport system associated with the plasma membrane of *Dictyostelium discoideum* is activated by different chemoattractant receptors. *J. Cell Biol.* **112**, 103-110.

Milne J. L., and Devreotes, P. N. (1993) The surface cyclic AMP receptors, cAR1, cAR2, and cAR3, promote Ca2+ influx in *Dictyostelium discoideum* by a G alpha 2-independent mechanism. *Mol. Biol. Cell* **4**, 283-292.

Milne, J. L., Kim, J. Y., and Devreotes, P. N. (1997) Chemoattractant receptor signaling: G protein-dependent and -independent pathways. *Adv. Second Messenger Phosphoprotein Res.* **31**, 83-104.

Meredith, M. J., and Reed, D. J. (1982) Status of the mitochondrial pool of glutathione in the isolated hepatocyte. *J. Biol. Chem.* **257**, 3747-3753.

Morita, T., Amagai, A., and Maeda, Y. (2004) Translocation of the *Dictyostelium* TRAP1 homologue to mitochondria induces a novel prestarvation response. *J. Cell Sci.* **117**, 5759-5770.

Mutzel, R., Lacombe, M.L., Simon, M.N., De Gunzburg, J., and Veron, M. (1987) Cloning and cDNA sequence of the regulatory subunit of cAMP-dependent protein kinase from *Dictyostelium discoideum*. *Proc. Natl. Acad. Sci. U.S.A.* **84**, 6–10.

Newton, G. L., and Fahey, R. C. (1995) Determination of biothiols by bromobimane labeling and high-performance liquid chromatography. *Methods Enzymol.* **251**, 148-166.

Noctor, G., Mhamdi, A., Chaouch, S., Han, Y., Neukermans, J., Marquez-Garcia, B., Queval, G. and Foyer, C. H. (2012) Glutathione in plants: an integrated overview. *Plant Cell Environ.* **35**, 454-484.

Otsuka, H., and Van Haastert, P. J. (1998) A novel Myb homolog initiates *Dictyostelium* development by induction of adenylyl cyclase expression. *Genes Dev.* **12**, 1738-1748.

Pang, K. M., Lynes, M. A., and Knecht, D. A. (1999) Variables controlling the expression level of exogenous genes in *Dictyostelium*. *Plasmid* **41**, 187-197.

Parent, C. A., and Devreotes, P. N. (1996) Constitutively active adenylyl cyclase mutant requires neither G proteins nor cytosolic regulators. *J. Biol. Chem.* **271**, 18333-18336.

Pasternak, M., Lim, B., Wirtz, M., Hell, R., Cobbett, C. S., and Meyer, A. J. (2008) Restricting glutathione biosynthesis to the cytosol is sufficient for normal plant development. *Plant J.* **53**, 999-1012.

Pitt, G. S., Milona, N., Borleis, J., Lin, K. C., Reed, R. R., and Devreotes, P. N. (1992) Structurally distinct and stage-specific adenylyl cyclase genes play different roles in *Dictyostelium* development. *Cell* **69**, 305-315.

Raper, K. B. (1940) Pseudoplasmodium formation and organization in Dictyostelium discoideum. *J. Elisha Mitchell Sci. Soc.* **56**, 241-282.

Rathi, A., and Clarke, M. (1992) Expression of early developmental genes in *Dictyostelium discoideum* is initiated during exponential growth by an autocrine-dependent mechanism. *Mech. Dev.* **36**, 173-182.

Rathi, A., Kayman, S. C., and Clarke, M. (1991) Induction of gene expression in *Dictyostelium* by prestarvation factor, a factor secreted by growing cells. *Dev. Genet.* **12**, 82-87.

Reid, A.B., Kurten, R.C., McCullough, S.S., Brock, R.W., and Hinson, J.A. (2005) Mechanisms of acetaminophen-induced hepatotoxicity: role of oxidative stress and

mitochondrial permeability transition in freshly isolated mouse hepatocytes. *J. Pharmacol. Exp. Ther.* **312**, 509-516.

Sambrook, J. and Gething, M.J. (1989) Protein structure. Chaperones, paperones. *Nature* **342**, 224-225.

Saran, S., and Schaap, P. (2004) Adenylyl cyclase G is activated by an intramolecular osmosensor. *Mol. Biol. Cell* **15**, 1479-1486.

Saraste, M., Sibbald, P. R., and Wittinghofer, A. (1990). The P-loop—a common motif in ATP- and GTP-binding proteins. *Trends Biochem. Sci.* **15**, 430-434.

Sarbassov, D. D., Ali, S. M., Kim, D. H., Guertin, D. A., Latek, R. R., Erdjument-Bromage, H., Tempst, P., and Sabatini, D. M. (2004) Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. *Curr. Biol.* **14**, 1296-1302.

Saxe, C. 3rd., Ginsburg, G. T., Louis, J. M., Johnson, R., Devreotes, P. N., and Kimmel, A. R. (1993) CAR2, a prestalk cAMP receptor required for normal tip formation and late development of *Dictyostelium discoideum*. *Genes Dev.* **7**, 262-272.

Saxe, C. L. 3rd., Johnson, R., Devreotes, P. N., and Kimmel, A. R. (1991) Multiple genes for cell surface cAMP receptors in *Dictyostelium discoideum*. *Dev. Genet.* **12**, 6-13.

Schlatterer, C., Knoll, G. and Malchow, D. (1992). Intracellular calcium during chemotaxis of *Dictyostelium discoideum*: a new fura-2 derivative avoids sequestration of the indicator and allows long-term calcium measurements. *Eur. J. Cell Biol.* **58**, 172-181.

Schulkes, C., and Schaap, P. (1995) cAMP-dependent protein kinase activity is essential for preaggregative gene expression in *Dictyostelium*. *FEBS Lett.* **368**, 381-384.

Schnitzler, G.R., Briscoe, G., Brown, J.M., and Firtel, R.A. (1995) Serpentine cAMP receptors may act through a G-protein-independent pathway to induce post-aggregative development in *Dictyostelium*. *Cell* **81**, 735-745.

Segall, J. E., Kuspa, A., Shaulsky, G., Ecke, M., Maeda, M., Gaskins, C., Firtel, R. A., and Loomis, W. F. (1995) A MAP kinase necessary for receptor-mediated activation of adenylyl cyclase in *Dictyostelium*. *J. Cell Biol.* **128**, 405-413.

Shaulsky, G., and Loomis, W. F. (1995) Mitochondrial DNA replication but no nuclear DNA replication during development of *Dictyostelium*. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 5660-5663.

Schatzle, J., Bush, J., and Cardelli, J. (1992) Molecular cloning and characterization of the structural gene coding for the developmentally regulated lysosomal enzyme, α-mannosidase, in *Dictyostelium discoideum*. *J. Biol. Chem.* **267**, 4000-4007.

Sherr, C. J. (1996) Cancer cell cycles. *Science* **274**, 1672-1677.

Shi, Z. Z., Osei-Frimpong, J., Kala, G., Kala, S. V., Barrios, R. J., Habib, G. M., Lukin, D. J., Danney, C. M., Matzuk, M. M., and Lieberman, M. W. (2000) Glutathione synthesis is essential for mouse development but not for cell growth in culture. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 5101-5106.

Sies, H. (1999) Glutathione and its role in cellular functions. *Free Radical Biol. Med.* **27**, 916-921.

Simon, M. N., Pelegrini, O., Véron, M., and Kay, R. R. (1992) Mutation of protein kinase A causes heterochronic development of *Dictyostelium*. *Nature* **356**, 171-172.

Soll, D.R., Yarger, J. and Mirick, M. (1976) Stationary phase and the cell cycle of *Dictyostelium discoideum* in liquid nutrient medium. *J. Cell Sci.* **20**, 513-523.

Souza, G. M., Hirai, J., Mehta, D. P., and Freeze, H. H. (1995) Identification of two novel *Dictyostelium discoideum* cysteine proteinases that carry *N*-acetylglucosamine-1-P modification. *J. Biol. Chem.* **270**, 28938-28945.

Souza, G. M., Lu, S., and Kuspa, A. (1998) YakA, a protein kinase required for the transition from growth to development in *Dictyostelium*. *Development* **125**, 2291-2302.

Souza, G. M., da Silva, A. M., and Kuspa, A. (1999) Starvation promotes *Dictyostelium* development by relieving PufA inhibition of PKA translation through the YakA kinase pathway. *Development* **126**, 3263-3274.

Sun, T. J., and Devreotes, P. N. (1991) Gene targeting of the aggregation stage cAMP receptor cAR1 in *Dictyostelium*. *Genes Dev.* **5**, 572-582.

Sun, T. J., Van Haastert, P. J., and Devreotes, P. N. (1990) Surface cAMP receptors mediate multiple responses during development in *Dictyostelium*: evidenced by antisense mutagenesis. *J. Cell Biol.* **110**, 1549-1554.

Sussman, M. (1987) Cultivation and synchronous morphogenesis of *Dictyostelium* under controlled experimental conditions. *Methods Cell Biol.* **28**, 9-29.

Taminato, A., Bagattini, R., Gorjão, R., Chen, G., Kuspa, A., and Souza, G. M. (2002) Role for YakA, cAMP, and protein kinase A in regulation of stress responses of *Dictyostelium discoideum* cells. *Mol. Biol. Cell* **13**, 2266-2275.

Thomas, D., Klein, K., Manavathu, E., Dimmock, J. R., and Mutus, B. (1991) Glutathione levels during thermal induction of the yeast-to-mycelial transition in *Candida albicans*. *FEMS Microbiol. Lett.* **77**, 331-334.

Thomason, P. A., Traynor, D., Cavet, G., Chang, W. T., Harwood, A. J., and Kay, R. R. (1998) An intersection of the cAMP/PKA and two-component signal transduction systems in *Dictyostelium*. *EMBO J.* **17**, 2838-2845.

Townsend, D. M., Tew, K. D., and Tapiero, H. (2003) The importance of glutathione in human disease. *Biomed. Pharmacotherap.* **57**, 145-155.

Van Driessche, N., Shaw, C., Katoh, M., Morio, T., Sucgang, R., Ibarra, M., Kuwayama, H., Saito, T., Urushihara, H., Maeda, M., Takeuchi, I., Ochiai, H., Eaton, W., Tollett, J., Halter, J., Kuspa, A., Tanaka, Y., and Shaulsky, G. (2002) A

transcriptional profile of multicellular development in *Dictyostelium discoideum*. *Development* **129**, 1543-1552.

van Es, S., Virdy, K. J., Pitt, G. S., Meima, M., Sands, T. W., Devreotes, P. N., Cotter, D. A., and Schaap, P. (1996) Adenylyl cyclase G, an osmosensor controlling germination of *Dictyostelium* spores. *J. Biol. Chem.* **271**, 23623-23625.

Van Haastert P. J. (1995) Transduction of the chemotactic cAMP signal across the plasma membrane of *Dictyostelium* cells, *Experientia* **51**, 1144-1154.

Van Lookeren Campagne, M. M., Franke, J., and Kessin, R. H. (1991) Functional cloning of a *Dictyostelium discoideum* cDNA encoding GMP synthetase. *J. Biol. Chem.* **266**, 16448-16452.

Vernoux, T., Wilson, R. C., Seeley, K. A., Reichheld, J. P., Muroy, S., Brown, S., Maughan, S. C., Cobbett, C. S., Van Montagu, M., Inze, D., May, M. J., and Sung, Z. R. (2000) The ROOT MERISTEMLESS1/CADMIUM SENSITIVE2 gene defines a glutathione-dependent pathway involved in initiation and maintenance of cell division during postembryonic root development. *Plant Cell* **12**, 97-109.

Viña, J., Saez, G. T., Wiggins, D., Roberts, A. F., Hems, R., and Krebs, H. A. (1983) The effect of cysteine oxidation on isolated hepatocytes. *Biochem. J.* **212**, 39-44.

Wang, B., and Kuspa, A. (2002) CulB, a putative ubiquitin ligase subunit, regulates prestalk cell differentiation and morphogenesis in *Dictyostelium spp*. *Eukaryot. Cell* **1**, 126-136.

Weeks, G., and Weijer, C. J. (1994) The *Dictyostelium* cell cycle and its relationship to differentiation. *FEMS Microbial. Lett.* **124**, 123-130.

Wharton, R. P., Sonoda, J., Lee, T., Patterson, M., and Murata, Y. (1998) The Pumilio RNA-binding domain is also a translational regulator. *Mol. Cell* **1**, 863-872.

Winkler, A., Njålsson, R., Carlsson, K., Elgadi, A., Rozell, B., Abraham, L., Ercal, N., Shi, Z. Z., Lieberman, M. W., Larsson, A., and Norgren, S. (2011) Glutathione

is essential for early embryogenesis – Analysis of a glutathione synthetase knockout mouse. *Biochem. Biophys. Res. Commun.* **412**, 121-126.

Williams, J. (1995) Morphogenesis in *Dictyostelium*: new twists to a not-so-old tale. *Curr. Opin. Genet. Dev.* **5**, 426-431.

Wu, L., Hansen, D., Franke, J., Kessin, R. H., and Podgorski, G. J. (1995) Regulation of *Dictyostelium* early development genes in signal transduction mutants. *Dev. Biol.* **171**, 149-158.

Wu, L., Valkema, R., Van Haastert, P. J., and Devreotes, P. N. (1995) The G protein beta subunit is essential for multiple responses to chemoattractants in Dictyostelium. *J.Cell Biol.* **129**, 1667-1675.

Wu, Y., Zhang, X., Bardag-Gorce, F., Robel, R. C., Aguilo, J., Chen, L., Zeng, Y., Hwang, K., French, S. W., Lu, S. C., and Wan, Y. J. (2004) Retinoid X receptor α regulates glutathione homeostasis and xenobiotic detoxification processes in mouse liver. *Mol. Pharmacol.* **65**, 550-557.

Yuen, I. S., Jain, R., Bishop, J. D., Lindsey, D. F., Deery, W. J., Van Haastert, P. J., and Gomer, R. H. (1995) A density-sensing factor regulates signal transduction in *Dictyostelium*. *J. Cell Biol.* **129**, 1251-1262.

Zamore, P. D., Bartel, D. P., Lehmann, R., and Williamson, J. R. (1999) The PUMILIO-RNA interaction: a single RNA-binding domain monomer recognizes a bipartite target sequence. *Biochemistry* **38**, 596-604.

Zhang, H., and Forman, H. J. (2012) Glutathione synthesis and its role in redox signaling. *Semin. Cell Dev. Biol.* **23**, 722-728

Zhang, B., Gallegos, M., Puoti, A., Durkin, E., Fields, S., Kimble, J., and Wickens, M. P. (1997) A conserved RNA-binding protein that regulates sexual fates in the *C. elegans* hermaphrodite germ line. *Nature* **390**, 477-484.

Zhang, N., Long, Y., and Devreotes, P. N. (2001) Gγ in *Dictyostelium*: its role in localization of Gβγ to the membrane is required for chemotaxis in shallow gradients. *Mol. Biol. Cell* **12**, 3204-3213.

국문초록

Glutathione은 진핵생물의 세포 내에서 높은 농도로 존재하는 tripeptide로 –SH(thiol) 작용기를 가지고 있어 중요한 세포 내부의 반응에 참여한다. 선행연구결과에 따르면, glutathione은 세포의 성장과 분화 모두에 있어 중요한 작용을 한다. Glutathione을 합성할 수 없는 균주에서 세포 내 methylglyoxal의 축적으로 인한 세포의 성장저해와 세포사멸이 관찰되었고, 분화에 있어서도 glutathione없을 때에는 초기배아발생에 치명적인 결함을 유발해 배아의 정상적인 발생을 저해하는 것이 보고되어있다. Glutathione의 주요성은 많이 보고되고 있지만 정확한 작용기작에 대한 이해는 부족하다. 본 연구는 뚜렷하고 관찰하기 쉬운 분화형태를 가지고 있는 *Dictyostelium discoideum*을 이용하여, 세포 내 glutathione이 완전하게 제거되었을 때 분화에 어떤 영향을 미치는지를 확인하여 분화과정에서 glutathione의 역할을 규명하고자 하였다.

Glutathione을 합성할 수 없는 돌연변이주(GCS 결실균주)는 외부에서 glutathione을 추가적으로 넣어주지 않으면 분화를 시작하지 못하였다. 영양분이 고갈되면 *Dictyostelium*은 세포 외부의 cAMP의 농도를 인지하고 한 방향으로 모여들어 다세포의 군집형태를 이루는데, GCS 결실균주는 군집형태를 이루지 못하였다. GCS 결실균주의 분화양상은 액체 분화배지에서 분화를 유도시켜 더욱 자세히 관찰되었다. 야생균주는 세포들이 모여 하나의 세포군집을 이루었지만, GCS 결실균주는 계속해서 단일 세포의 상태로 존재하고 있었다. 이런 GCS 결실균주의 분화과정에서의 결함은 –thiol 작용기를 가지고 있는 다른 화학물질(dithiothreitol (DTT), *N*-

acetylcysteine (NAC))과 일반적인 항산화물질로 알려져 있는 ascorbic acid을 첨가해주어도 회복되지 않았다. 즉, *Dictyostelium*의 분과과정에서 glutathione은 세포내 산화·환원 환경을 조절하는 것뿐만 아니라 고유의 역할을 하고 있음을 알수 있었다.

*Dictyostelium*의 분화는 많은 유전자들의 발현을 정밀하게 조절하여 적절하게 일어나는 일련의 과정들이다. 분화가 시작되면, 세포성장에 관여하던 유전자(*cprD*)들의 발현은 감소되고 분화에 필요한 유전자들의 발현은 크게 증가한다. 그 중에서도 cAMP신호전달에 관여하는 단백질 유전자들인 *carA*와 *acaA*의 발현은 분화초기단계 조절에 중요한 역할을 한다. GCS 결실균주에서는 *cprD*의 발현이 증가되어 있고, *carA*와 *acaA*의 발현이 현저하게 감소되어있었다. 외부에서 cAMP를 첨가해 자극해주거나 cAMP를 인식하는 단백질(cAR1)을 과량발현시켜 cAMP 신호전달을 인위적으로 유도하여도 glutathione의 첨가 없이는 분화하지 못하였다. 즉, *Dictyostelium*의 분화에서 glutathione은 cAMP에의한 신호전달체계가 작용하는 시기보다 더 앞선 단계에 작용할 것으로 생각된다.

GCS 결실균주에서는 YakA의 유전자가 발현되지 않았다. 또한 *yakA*의 발현양은 세포 내부의 glutathione의 농도에 비례하여 증가하는 것이 관찰되었다. 그리고 분화형태나 분화초기에 중요한 역할을 하는 것으로 알려져 있는 유전자들의 발현양상이 YakA 결실균주와 GCS 결실균주에서 유사하였다. YakA를 GCS 결실균주에 과량발현시키면, glutathione이 없어도 분화하여 다세포성 세포군집을 형성하였고, 분화초기에 관여하는 것으로 알려져있는 유전자들또한 정상적으로 발현되었다. GCS 결실균주의 분화결함은 YakA를 발현시켜줌으써 회복되었지만, YakA 결실균주는 glutathione을

첨가해주어도 분화를 하지 못하였다. 또한 YakA 과량발현에 의해 세포 내 glutathione 농도나 *gcsA*의 발현양이 크게 영향을 받지 않는 것으로 보아 YakA가 세포 내 glutathione의 농도를 직접적으로 조절하는 것은 아닌 것으로 생각된다.

위의 결과를 종합하여 보았을 때, 세포 내 glutathione은 영양분의 고갈과 같은 분화 환경조건에 반응하여 YakA 유전자의 발현을 유도하고, 그 하위단계 작용단백질들의 발현을 조절하여 YakA 신호전달을 활성화 시킴으로써 *Dictyostelium*의 세포성장에서 분화로의 전환을 조절하는 것으로 생각된다.

주요어; Glutathione, YakA, 분화, *Dictyostelium discoideum*

理學博士學位論文

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Roles of glutathione in differentiation of *Dictyostelium discoideum*

by

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ABSTRACT

Reduced glutathione (GSH, γ-L-glutamyl-L-cysteinylglycine) is a ubiquitous tripeptide found in almost all organisms and the most abundant non-protein thiol-containing compound in eukaryotic cells. It is known to participate in diverse cellular functions, such as antioxidant defenses, the regulation of intracellular redox status, signal transduction, cell proliferation and death, and immune responses. GSH also participates in regulation of organ differentiation.

Previously, it was reported that GSH serves important roles in normal growth and differentiation of *Dictyostelium discoideum*. The developmental morphology of g*csA*$^{-}$ cells was dependent on the concentration of GSH which was added to culture media. In this work, to find out the precise roles of GSH during development, intracellular GSH was completely depleted and then developmental morphology was observed. Absence of GSH caused defects in the formation of multicellular aggregates. g*csA*$^{-}$ cells were in a state of single cells if GSH was not supplemented. This developmental defect of g*csA*$^{-}$ cells was rescued by adding exogenous GSH, γ-GC, or GSSG. But other thiol-compounds or antioxidant molecules, such as DTT, NAC, and ascorbic acid, did not compensate GSH. These results indicate that GSH itself plays essential roles rather than as an antioxidant molecule in regulating the development of *Dictyostelium*.

To gain more information on the developmental defect of *gcsA*$^{-}$ cells, the expression patterns of genes that were required to initiate development were examined. GSH-depleted *gcsA*$^{-}$ cells failed to decrease the expression of a growth-stage-specific gene (*cprD*) and failed to induce the expression of genes

that encode proteins required for early development (discoidin, *dscA*; differentiation associated protein, *dia2*; cAMP receptor, *carA*/cAR1; adenylyl cyclase, *acaA*/ACA; and the catalytic subunit of protein kinase A, *pkaC*/PKA-C). Decreased expression of *carA* and *acaA* was remarkable in *gcsA*$^{-}$ cells. However, the developmental defect of *gcsA*$^{-}$ cells was not restored by cAMP stimulation or by cAR1 expression. Though constitutively expressed cAR1 induced the expression of *acaA* and Gα2 gene, *gcsA*$^{-}$ cells did not develop without GSH. These results suggest that GSH seems to work at higher step to the cAMP signaling pathway to regulate development of *Dictyostelium*.

YakA signaling is known the earliest response to environmental signal to initiate development and functions prior to cAMP signaling. The expression of *yakA* is responsible to induce the expression of differentiation-associated genes and to inhibit the expression of growth-phase genes for the initiation of development. The expression of *yakA* was regulated by intracellular GSH in both KAx3 and *gcsA*$^{-}$ cells. GSH-depleted *gcsA*$^{-}$ cells showed undetectably low *yakA* expression levels, but the expression was induced by adding GSH. The expression of *yakA* was in proportion to the concentration of exogenously added GSH in KAx3 cell. Further, induced *yakA* expression promoted the formation of multicellular aggregate in both KAx3 and *gcsA*$^{-}$ cells. Intracellular GSH also influenced on the expression of *pufA* and the activity of PKA, which are components of downstream regulators in the YakA signaling pathway. *gcsA*$^{-}$ cells showed increased *pufA* expression and lowered PKA activity compared to KAx3 cells. However, the expression of *pufA* and the activity of PKA were recovered to the similar level of KAx3 cells by adding GSH. Interestingly, *yakA*$^{-}$ cells showed similar gene expression pattern and developmental morphology to *gcsA*$^{-}$ cells. *yakA*$^{-}$ cells did not develop. The expression of *carA*

and *acaA* was significantly decreased and the activity of PKA was not detected in *yakA*$^{-}$ cells. Exogenous GSH did not rescue the developmental defects of *yakA*$^{-}$ cells, but constitutively expressed YakA in *gcsA*$^{-}$ cells (YakA^{OE}/*gcsA*$^{-}$) rescued the developmental defects of *gcsA*$^{-}$ cells without the addition of GSH; YakA^{OE}/*gcsA*$^{-}$ cells formed multicellular aggregates and *carA* and *acaA* were expressed without GSH. These results indicate that intracellular GSH plays indispensable roles during development by regulating the expression of *yakA* in *Dictyostelium*.

To investigate the relation between YakA and GSH further, the concentration of intracellular GSH the expression of *gcsA* were monitored in *yakA*$^{-}$ and YakA^{OE}/KAx3 cells. *yakA*$^{-}$ cells showed decreased intracellular GSH levels around 40% compared to KAx3 and considerably increased *gcsA* expression. However, constitutive expression of YakA in KAx3 cells (YakA^{OE}/KAx3 cells) did not significantly influence on the intracellular GSH level and *gcsA* expression, indicating that GSH regulates the expression of *yakA* but YakA did not regulate intracellular GSH. Decreased intracellular GSH concentration might be caused by hypersensitiveness to oxidative stress of *yakA*$^{-}$ cells and leads to accumulation of *gcsA* transcripts by the feedback regulation of GSH.

Taken together, these findings suggest that GSH plays an essential role in the transition from growth to differentiation by modulating the expression of the genes encoding YakA as well as components that act downstream in the YakA signaling pathway in *Dictyostelium*.

Key words: Glutathione; YakA; Transition from growth to differentiation; *Dictyostelium discoideum*

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LIST OF ABBREVIATIONS

GSH	reduced glutathione
GSSG	oxidized glutathione
γ-GC	γ-glutamylcysteine
GCS	γ-glutamylcysteine synthetase
GSS	glutathione synthetase
ROS	reactive oxygen species
PSF	prestarvation factor
CMF	conditioned medium factor
cAMP	3′-5′-cyclic adenosine monophosphate
cAR1	cAMP receptor
ACA	adenylyl cyclase
PKA	cAMP-dependent protein kinase A
bp	base pair
Da	dalton
rpm	revolutions per minute
RT	reverse transcriptase
PCR	polymerase chain reaction
HPLC	high performance liquid chromatography
LB	Luria-Bertani
DTT	dithiothreitol
NAC	*N*-acetylcysteine
mBBr	monobromobimane
NEM	*N*-ethylmaleimide
SDS	sodium dodecyl sulfate
EDTA	ethylenediaminetetraacetate
DEPC	diethylpyrocarbonate

I. INTRODUCTION

1. Glutathione

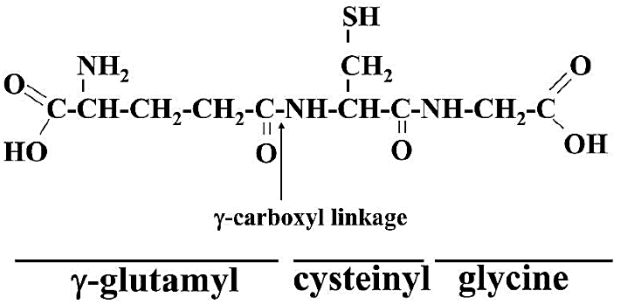
1.1. An overview

Glutathione (GSH) is a ubiquitous tripeptide, γ-L-glutamyl-L-cysteinylglycine (Scheme 1A), found in most plants, microorganisms, and all mammalian tissues (Meister and Anderson, 1983). It is the main derivative of cysteine and the most abundant intracellular non-protein thiol. Eukaryotic cells have three major reservoirs of GSH. Almost 90% of cellular GSH are in the cytosol, 10% in the mitochondria, and a small percentage in the endoplasmic reticulum (Meredith and Reed, 1982; Hwang *et al.*, 1992).

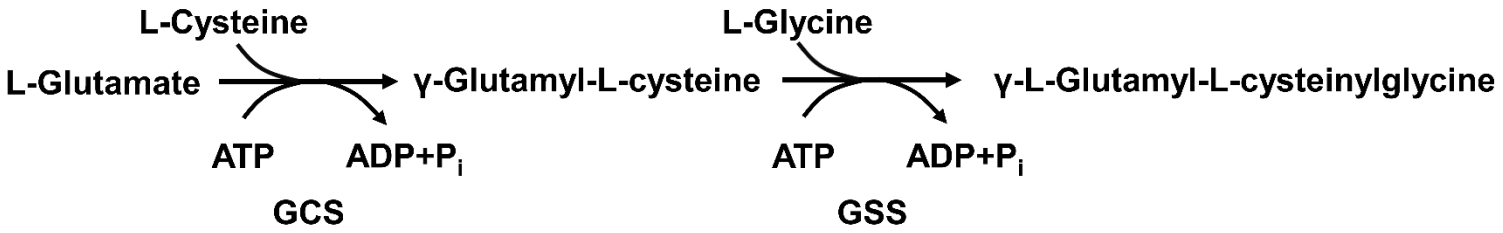
Glutathione exists in thiol-reduced form (GSH) and disulfide-oxidized (GSSG) form (Kaplowitz *et al.*, 1985). Under physiological conditions, most cellular constituents are reduced. In cells, glutathione is maintained in the reduced form (GSH) by the action of glutathione reductase and NAD(P)H. Because the oxidized form (GSSG) is efficiently reduced, the intracellular ratio of GSH to GSSG is high in most eukaryotic cells (Halliwell and Gutteridge, 1989). GSSG content is rarely exceeds 10% of total glutathione (Akerboom *et al.*, 1982; Halliwell and Gutteridge, 1989; Wu *et al.*, 2004). Maintaining optimal GSH:GSSG ratios in cells is critical to survival, hence, tight regulation of the system is imperative. The GSH to GSSG ratio is often used as an indicator of the cellular redox state.

The potent electron donating capacity of sulfhydryl group is the key to the multiple actions of GSH at the molecular, cellular and tissue level (Meister, 1994). The free sulfhydryl moiety of the cysteine residue confers high redox potential $E'_0 = -0.33$ V (Lewin, 1976). Its high negative redox potential renders GSH both a potent antioxidant and a convenient cofactor

A



B



Scheme 1. Chemical structure and enzymatic synthesis of GSH (γ-L-Glutamyl-L-cysteinylglycine).

(A) Chemical structure of GSH. (B) Enzymatic biosynthesis of GSH. GCS; γ-glutamylcysteine synthetase, GSS; glutathione synthetase.

for enzymatic reactions that require readily available electron pairs. Intracellular stability, which is promoted by the exceptional γ-glutamyl linkage and lack of the toxicity associated with cysteine (Vina *et al.*, 1983), make GSH suitable as a cellular thiol redox buffer to maintain a thiol/disulfide redox potential.

In cells, tissues, and plasma, glutathione is present in several additional forms. Glutathione disulfide (GSSG) is formed upon oxidation. Other forms of disulfide are of the mixed type, GSSR, a major class of biologically interesting ones being glutathione-cysteinyl disulfides on proteins.

1.2. The enzymatic synthesis of glutathione

The synthesis of GSH from glutamate, cysteine, and glycine is catalyzed sequentially by two cytosolic enzymes, γ-glutamylcysteine synthetase (GCS) and GSH synthetase (GSS) (Scheme 1B).

In the GCS reaction, the γ-carboxyl group of glutamate reacts with the amino group of cysteine to form a peptide γ-linkage, which protects GSH from hydrolysis by intracellular peptidase. γ-Glutamylcysteine synthetase is rate-limiting enzyme in de novo synthesis of GSH (Meister, 1983). Induction of GCS expression has been demonstrated in response to diverse stimuli in a cell specific manner. The bioavailability of cysteine regulates the synthesis of GSH. Post-translational modification of GCS also influence GSH synthesis (Bella *et al.*, 1999; Gomi *et al.*, 1997). Specifically, phosphorylation of GCS leads to the inhibition of GSH synthesis. GSH itself regulates the activity of GCS via a negative feedback mechanism (Meister and Anderson, 1983). Hence, GSH depletion increases the rate of GSH

synthesis. The mechanistic links between feedback inhibition and thiol/disulfide redox regulation of GCS remain to be elucidated.

Mammalian GCS is a heterodimer consisting of a catalytically active heavy subunit (GCS_h, 73 kDa) and a light regulatory subunit (GCS_l, 31 kDa) (Lu, 2000). There is a variable degree of sequence identity among the cDNA sequences and the deduced amino acid sequences of the various eukaryotic GCS catalytic subunit proteins. The cDNA of mammalian and yeast cDNA sequences shows the highest degree of similarity (90-95%) (Griffith and Mulcahy, 1999). By contrast, the bacterial and Arabidopsis GCS encode catalytic proteins that are smaller than those of the other species examined and share only limited amino acid sequence identity (<10% and <20%, respectively) (Griffith and Mulcahy, 1999).

GCS from *Dictyostelium discoideum* is distinct from the general eukaryotic forms. It is a monomer with a subunit molecular mass of 75 kDa, encoded by a single gene. However, the deduced amino acid sequence of *Dictyostelium* GCS has considerable sequence similarity with the protein of *Drosophila melanogaster*, *Homo sapiens*, and *Schizosaccharomyces pombe*, approximately 48%, 47%, and 43%, respectively. Like other catalytic subunit of GCS, the catalytic center of the active site, Cys-256, and a common motif found in phosphate binding sites, glycine-rich loop (Gly-249, Gly-251, and Gly-253), are well conserved in *Dictyostelium* (Griffith and Mulcahy, 1999; Saraste *et al.*, 1990).

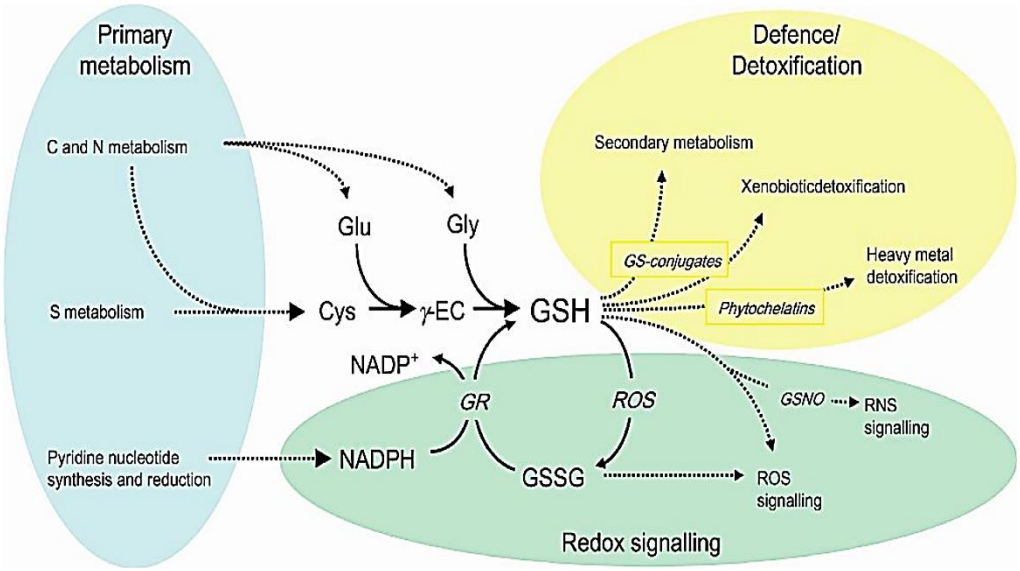
The second enzyme required for GSH biosynthesis is glutathione synthetase (GSS) (Scheme 1B). This enzyme functions as a homodimer of 118 kDa and is responsible for the addition of glycine to γ-glutamylcysteine created by GCS to form GSH. GSS is not subject to feedback inhibition by

GSH. In *Saccharomyces cerevisiae*, this enzyme is not essential for growth under both normal and oxidative stress conditions due to an accumulation of γ-glutamylcysteine, which protects against oxidative stress (Grant *et al.*, 1997). Overexpression of GSS failed to increase GSH level whereas overexpression of GCS increased the GSH level, consistent with the fact that GCS is the rate-limiting enzyme of GSH synthesis (Grant *et al.*, 1997).

1.3. The roles of glutathione in cellular reactions

Glutathione participates in many cellular reactions (Scheme 2). GSH displays remarkable metabolic and regulatory versatility. GSH/GSSG is the most important redox couple and plays crucial roles in an antioxidant defense, nutrient metabolism, and the regulation of pathways essential for whole cellular homeostasis.

First of all, GSH effectively scavenges free radicals and other reactive oxygen species (ROS) directly, and indirectly through enzymatic reactions (Grant *et al.*, 1996; Fang *et al.*, 2002) as part of the antioxidant barrier that prevents excessive oxidation of sensitive cellular components. In the mitochondria, GSH is particularly important because there is no catalase. Mitochondrial GSH is critical in defending against both physiologically and pathologically generated oxidative stress (Garcia-Ruiz and Fernandez-Checa, 2006). In such reactions, GSH is oxidized to form GSSG, which is then reduced to GSH by the NADPH-dependent glutathione reductase. Glutathione deficiency contributes to oxidative stress, and may play a key role in aging and the pathogenesis of many diseases. Decreased cellular levels of GSH have been observed in a number of diseases such as diabetes cancer, and HIV infection in which increased oxidative stress has been



Scheme 2. An integrated overview of the most important glutathione functions. Cys, cysteine; γ-EC, γ-glutamylcysteine; GS-conjugates, glutathione S-conjugates; GSNO, S-nitrosoglutathione; Glu, glutamate; Gly, glycine; RNS, reactive nitrogen species; ROS, reactive oxygen species (Noctor *et al.*, 2012).

implicated as the pathogenic metabolism (Townsend *et al.*, 2003).

Second, GSH maintains the intracellular redox balance and the essential thiol status of proteins (Lu, 1999). GSH undergoes thiol-disulfide exchange in a reaction catalyzed by thiol-transferase. As mentioned above, cellular GSSG content is extremely low so that protein mixed disulfide formation is limited. The thiol-disulfide equilibrium within the cell is known to regulate a diverse number of metabolic processes including enzyme activity, transport activity, signal transduction, and gene expression via alteration of redox sensitive transcription factors (Hutter *et al.*, 1997; Lu, 1999; Townsend *et al.*, 2003).

Third, GSH participates in cell signaling through at least two mechanisms, protein S-glutathionylation and cysteine S-nitrosylation (Zhang and Forman, 2012). These modifications change the conformation, stability, or activity of the target proteins. The former is formed when GSH conjugates with reactive cysteine residues within proteins. GSH also interact with nitric oxide (NO) system via formation of S-nitroglutathione (GSNO) (Lindermayr *et al.*, 2010). GSH may also indirectly participate in the redox signaling by changing cellular redox homeostasis (Sies, 1999).

Fourth, GSH regulates cell growth, proliferation, and cell death. Recent evidence suggests that an increased GSH level is associated with an early proliferative response and is essential for the cell to enter the S phase (Chaudhuri *et al.*, 1997; Lu, 2009; Aw, 2003). GSH modulates cell death at both extremes, apoptosis and necrosis, by regulating redox state of specific thiol residues of proteins such as NFκB, stress kinases, and caspases, involved in cell death (Galter *et al.*, 1994; Garcia-Ruiz and Fernandez-Checa, 2007). GSH depletion occurs during apoptosis in many different cell types,

secondary to increased reactive oxygen species (ROS), enhanced GSH efflux, and decreased GCS activity (Hall, 1999; Madeo *et al.*, 1999; Franklin *et al.*, 2003; Baek *et al.*, 2004).

Fifth, GSH functions in detoxification of xenobiotics or their metabolites (Ketterer *et al.*, 1983, Meister, 1994, Hayes and McLellan, 1999). GSH conjugates electrophilic those toxic compounds enzymatically or spontaneously in reactions catalyzed by GSH-S-transferase (Meister, 1988). The formed conjugates are usually excreted from the cell.

1.4. The roles of glutathione in development

The changes in redox environment during differentiation is provided by Allen *et al.* (1985) in a study of a slime mold (*Physarum polycephalum*). A sequential change in the antioxidant profile is also observed upon providing a stimulus for differentiation. As differentiation proceeded, superoxide dismutase (SOD) activity increases by as much as 21-fold. This increase in SOD activity parallels the rate of differentiation. In contrast, GSH concentration decreases during differentiation by more than 80% in all cultures, regardless of the initial concentration. The rate of differentiation is inversely related to the initial GSH concentration and directly proportional to the SOD activity. In sea urchin eggs, fluctuation of cellular thiols during development is also noted (Kawamura, 1960). Thomas *et al*. (1991) reported dramatic decrease in glutathione level during the thermal yeast-to-mycelial induction and suggested the potential involvement of intracellular glutathione levels in regulation of the morphogenesis in *Candida albicans*.

Recent studies have suggested an important role for GSH in mammalian development *in vitro* and *in vivo*. The embryo is exposed to increased

oxidative stress that exceeds the antioxidant defenses, resulting in a decrease in the GSH:GSSG ratio during subsequent development (Dumollard *et al.*, 2007). Glutathione levels increase during maturation of oocytes and subsequently decrease by 90% during early embryo development in blastocysts, in comparison to concentrations in mitotic stage oocytes (Gardiner and Reed, 1994; Luberda, 2005). The mechanism for this may involve ATP-dependent synthesis during oocyte maturation, which is switched off after fertilization. Pharmacologically induced GSH deficiency by an inhibitor (buthionine sulfoximine, BSO) in newborn mammals such as rats and guinea pig leads to rapid multi-organ failure and death within a few days (Meister, 1994). The generation of a null mutation of the heavy subunit of γ-glutamylcysteine synthetase results in complete GSH deficiency and caused embryo lethality in the mouse resulted from apoptotic cell death (Shi *et al.*, 2000; Winkler *et al.*, 2011).

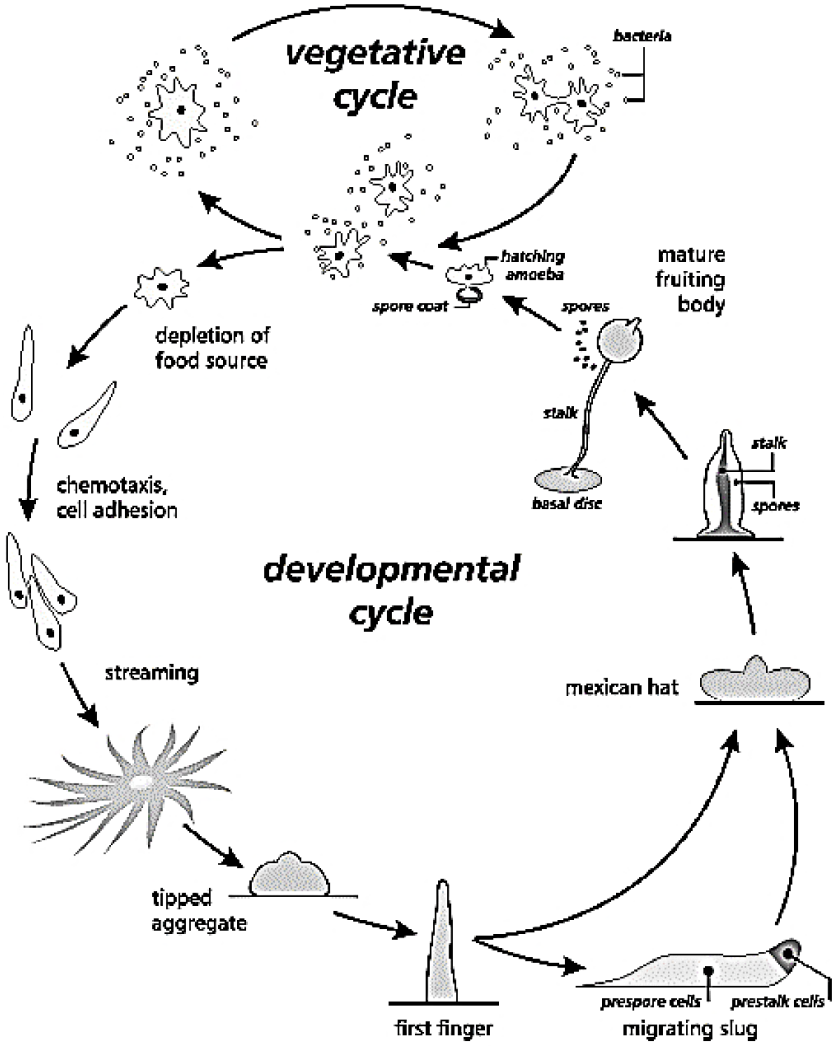
In plant cells, knocking out expression of GSH1, encoding the first enzyme of the committed pathway of GSH synthesis, causes lethality at the embryo stages (Cairns *et al.*, 2006; Noctor *et al.*, 2012), knockouts for GSH2, encoding glutathione synthetase, show a seedling-lethal phenotype (Pasternak *et al.*, 2008). In several mutants in which decreased GSH contents are caused by less severe mutation in the GSH1 gene. Of these mutants, which has less than 5% of wild-type glutathione contents, shows failure in development of a root apical meristem (Vernoux *et al.*, 2000). In other mutants, in which glutathione is decreased to about 25% to 50% of wild-type contents, developmental phenotypes are weak or absent, but alterations in environmental responses are observed.

2. *Dictyostelium discoideum*

2.1 Properties as a model organism

The cellular slime mold *Dictyostelium discoideum* is widely used to study multicellular morphogenesis. The life cycle of *Dictyostelium discoideum* comprises two phases. During the vegetative phase, cells grow as solitary amoebae and feed on bacteria in the soil and multiply by simple binary fission. As the food source becomes steadily depleted and the population increases, cells stop growing and initiate a coordinated developmental program that ultimately leads to the formation of a multicellular organism with only two main cell types, vacuolated stalk and dormant spore cells (Loomis, 1982; Firtel, 1995). Coordinated cell type differentiation and morphogenesis lead to a final fruiting body that allows the dispersal of spores which survive harsh environmental conditions (Scheme 3).

Upon starvation, cAMP is synthesized and released in nanomolar and a pulsatile manner from aggregation centers and attracts neighboring cells to migrate to the center. The pulsatile release of cAMP results in concentric or spiral-rings of amoebae which is called hemispherical mounds up to 100,000 cells that become enclosed in a protein cellulose slime sheath to form tight aggregates. A rise in the cAMP concentration to micro molar levels occurs (Abe and Yanagisawa, 1983), which initiates a developmental cascade (Schnitzler *et al.*, 1995). A protruding tip then forms at the apes of each aggregates. The tip behaves as an organizer, which are orchestrating all subsequent movements of developing cells (Raper, 1940; Durston, 1976; MacWilliams, 1982). Subsequently, once a tip has formed, the aggregate



Scheme 3. Life cycle of *Dictyostelium discoideum*. The life cycle of *Dictyostelium discoideum* consists of distinct two different phase. When nutrients are available, cells grow vegetatively as single-celled amoeba. When nutrients are deprived, cells initiate developmental life cycle (http://www.dictyostelium.com/).

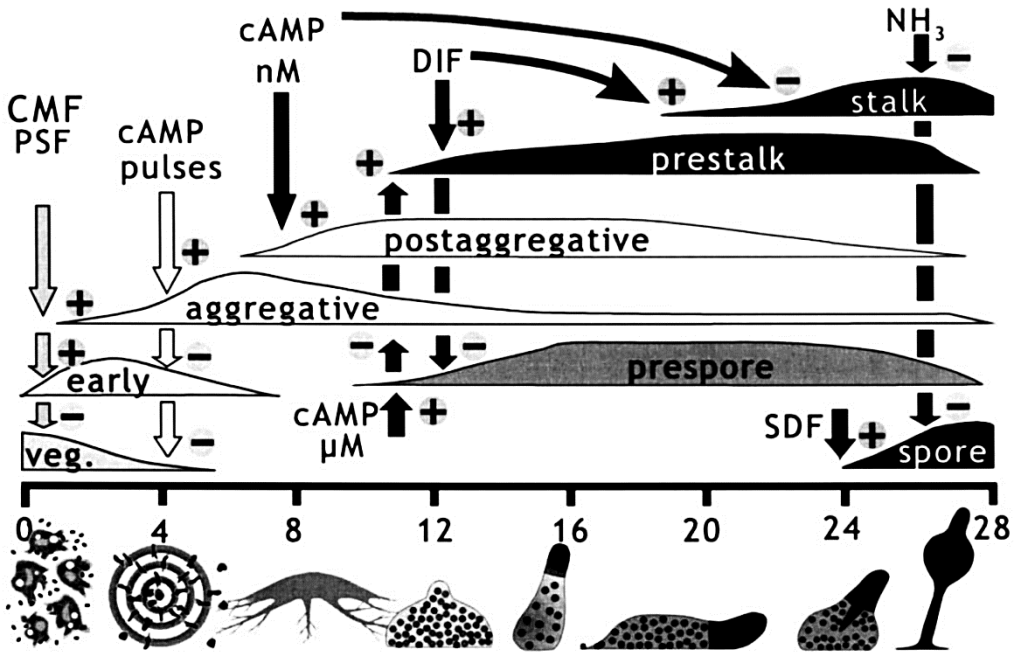
gradually elongates to give rise to an upright finger-like structure. In the finger and migrating slug, prestalk cells are localized to the anterior that are the precursors of the stalk cells of the mature fruiting body. Prespore cells are found in the posterior and the anterior-like cells (ALCs) which are scattered throughout slug (Gross, 1994). During culmination, prestalk cells penetrate through the prespore-cell mass and lift it off the substratum. Finally, cells mature into spore and stalk cells and form the final structure with a sorus atop a slender stalk (Loomis, 1982).

The life cycle of *Dictyostelium* cells is unique and relatively simple, but it contains almost all of the cellular processes such as cell movement, chemotaxis, cell adhesion, cell type determination, pattern formation, etc., essential for the establishment of multicellular organization. The 34 Mb genome contains many genes predicted to encode 125,000 proteins that are homologous to those in higher eukaryotes and are missing in *Saccharomyces cerevisiae*. Thus, *Dictyostelium* is a powerful system for genetic and functional analysis of gene function. The availability of biochemical and molecular genetics techniques has allowed the discovery of complex signaling networks which are important for *Dictyostelium* development and are also conserved in other organisms. The recent completion of the *Dictyostelium* genome sequence (Eichinger *et al.*, 2005; and accessible with related on-line resources at http://dcitybase.org) greatly facilitates such analysis. This relative simplicity has made *Dictyostelium* a model system for studying eukaryotic signal transduction and cell to cell communication during differentiation (Devreotes, 1994; Firtel, 1995; Williams, 1995).

2.2 The transition from growth to development

The process of transition from growth to development is of general importance for the development of organisms. Growth and differentiation are mutually exclusive, but they are cooperatively regulated during the course of development (Maeda, 2005). Similarly to most higher eukaryotic cells, the transition is regulated by complex molecular mechanisms, designed to ensure that development only occurs under optimal conditions in *Dictyostelium* (Scheme 4). Extracellular signals control the transition from growth to development and the changes are controlled by the activities of numerous regulators (William *et al.*, 1993; Katoh *et al.*, 2007). Cells prepare for future starvation and development by sensing environmental conditions and accumulating transcripts of a number of genes. Upon starvation, the expression of vegetative genes is reduced, whereas genes required for development are induced. When nutrients are depleted, cells stop replicating chromosomal DNA and reduce the expression of vegetative genes. However, the expression of genes required for development is induced to trigger aggregation.

Recent studies of gene transcription profiles show that aggregation of unicellular amoebae to multicellular structures is accompanied by a change in the expression of more than 25% of the genes in the genome (Van Driessche *et al.*, 2002). For example, the expression of V4 transcripts is induced by starvation. Antisense inhibition of V4 expression leads to a failure to inhibit the transcription of vegetative stage genes, and also leads to a reduction in the transcription of genes that are involved in the events of chemotaxis to cAMP (McPherson and Singleton, 1992). Among the genes that are repressed during early development, the transcription of several ribosomal protein genes has been rapidly reduced after starvation begins



Scheme 4. Regulation of gene expression during development of *Dictyostelium discoideum.* The transition from growth to development is regulated by modulating gene expression designed to ensure that aggregation occurs under optimal conditions. CMF, conditioned medium factor; PSF, prestarvation factor; DIF, differentiation inducing factor; SDF, spore differentiation factor; veg., vegetative. (Williams *et al.*, 1993)

(Ken and Singleton, 1994). The transcripts of several biosynthetic genes also disappear. These include transcripts for *cprD*, a growth stage cysteine proteinase (Souza et al., 1995), *pyr56*, UMP-synthetase, and *guaA*, GMP-synthetase (Jacquet *et al.*, 1988; Van Lookeren Campagne *et al.*, 1991).

The synthesis of most proteins are decreased in the hours after starvation, but the synthesis of several proteins is transiently induced. Abrupt changes in environment and starvation caused an immediate unloading of mRNA from polysomes and an increase in monomeric ribosomes (Margolskee and Lodish, 1980).

The cell cycle cessation is the major event during starvation, however mitochondrial DNA synthesis continues (Shaulsky and Loomis, 1995). As cell proliferation is finely regulated by extracellular signals such as growth factors, there are some checkpoints monitoring the exact progression of cell cycle. It has been shown that a specific checkpoint regulating the transition from growth to development in tumor cell exists in the G1 phase (Sherr, 1996). Although the cell cycle of *Dictyostelium* is regulated by the same components that regulate yeast or other cell cycles (Weeks and Weijer, 1994), it is not known that how it is shut off during starvation. The elucidation of relations between developmental signals and their pathway toward the regulations of genetic program must provide insights into general mechanisms for the initiation of cell differentiation.

2.3. Intracellular signals required for the initiation of development

During the growth phase, *Dictyostelium* cells continuously synthesize and secrete autocrine factors that accumulate in a cell-density-dependent

manner. At appropriate concentrations these factors induce changes in gene expression and prepare cells for the initiation of development. There are two density-sensing mechanisms that function during the early stages of development. One mechanism is the prestarvation response which is mediated by several prestarvation factors (PSFs) and that controls induction of certain very early genes (Rathi and Clarke, 1992). The other mechanism, mediated by conditioned medium factors (CMFs) (Gomer *et al.*, 1991; Iijima *et al.*, 1995), helps the cells to assess their density at a slightly later stage during aggregation. Those diffusible factors secreted during the growth or early development phase work as intercellular communicators that enables starving *Dictyostelium* cells to develop properly.

2.3.1. Prestarvation factors

Prestarvation factors (PSFs) are glycoproteins with a mass of 65-70 kDa and are sensitive to proteases and to heat. PSFs are synthesized during growth and accumulate in the microenvironment according to the density of the cells. Cells can detect the levels of PSFs secreted by growing cells and thus estimate their own density relative to the abundance of external nutrients (Clarke *et al.*, 1988; Maeda and Iijima, 1992; Morita *et al.*, 2004). The prestarvation response occurs during increases in PSFs levels and decreases in nutrients. PSFs regulates the expression of genes involved in sensing cAMP for the oncoming process of aggregation. For example, the expression of *pdsA*, which encodes the secreted cyclic nucleotide phosphodiesterase (ePDE) is induced by PSFs (Lacombe *et al.*, 1986). The ePDE is responsible for resetting the gradient sensing machinery and allowing cells to respond to additional chemotactic signals by degrading extracellular cAMP. The

expression of genes involved in aggregation such as members of the discoidin I gene family, cell adhesion molecule gp24, and lysosomal protein α-mannosidase gene (*manA)* is also induced by PSFs (Clarke *et al.*, 1987; Schatzle *et al.*, 1992). The expression of *carA* encoding the major cAMP receptor during early development is also regulated in this manner (Louis *et al.*, 1993; Rathi *et al.*, 1991; Sun and Devreotes, 1991). The gene inductive effect of PSF is inhibited by the presence of bacteria or the presence of nutrient source. Although the receptor of PSFs has not been identified, the response has been shown to partly depend on signaling to G proteins. In the G protein-dependent pathway, the folate released from bacteria downregulates PSF signaling (Mahadeo and Parent, 2006). The synthesis of PSF declines as development proceeds. PSFs do not promote further development in the absence of starvation.

2.3.2. Conditioned medium factors

Other secreted glycoproteins, conditioned medium factors (CMFs), may play a role in the growth to development transition. When food is depleted, cells stop growing and activate the starvation responses by secreting CMFs. Secreted CMFs are needed to activate cAMP signaling and to initiate aggregation. (Mann and Firtel, 1989; Gomer *et al.*, 1991; Yuen *et al.*, 1995). CMFs are glycoproteins with a molecular mass of 80 kDa (Gomer *et al.*, 1991). Growth-phase cells are able to synthesize CMFs but do not secrete it to growth medium. Upon exhaustion of nutrients, however, starved cells simultaneously secret and sense CMFs to monitor the local cell density during early differentiation. Just before the aggregation stage, CMFs continuously induce early developmental genes such as *discoidin I* and

mediate the expression of a number of early developmentally regulated genes for cAMP pulses and chemotaxis. According to Yuen *et al*. (1995), although cAMP receptors are present in the absence of CMF, the responses to cAMP pulses such as the activation of Ca^{2+} influx, adenylyl cyclase, and guanyly cyclase are strongly inhibited in cells lacking CMFs. The activations are restored by exposure to exogenous recombinant CMFs. The activation of phospholipase C (PLC) by cAMP pulses is not affected by the presence of CMFs (Yuen *et al*., 1995). The interaction of the cAMP receptor with G proteins is also not affected by CMF. However, the activation of adenylyl cyclase by GTPγS requires cell to have been exposed to CMFs, indicating that CMFs controls cAMP signal transduction. CMFs regulates cell aggregation by mediating cAMP signaling at a step after cAMP induces Gα2 to exchange GDP for GTP, but before Gα2 GTP activates adenylyl cyclase.

2.4. The early events induced by starvation

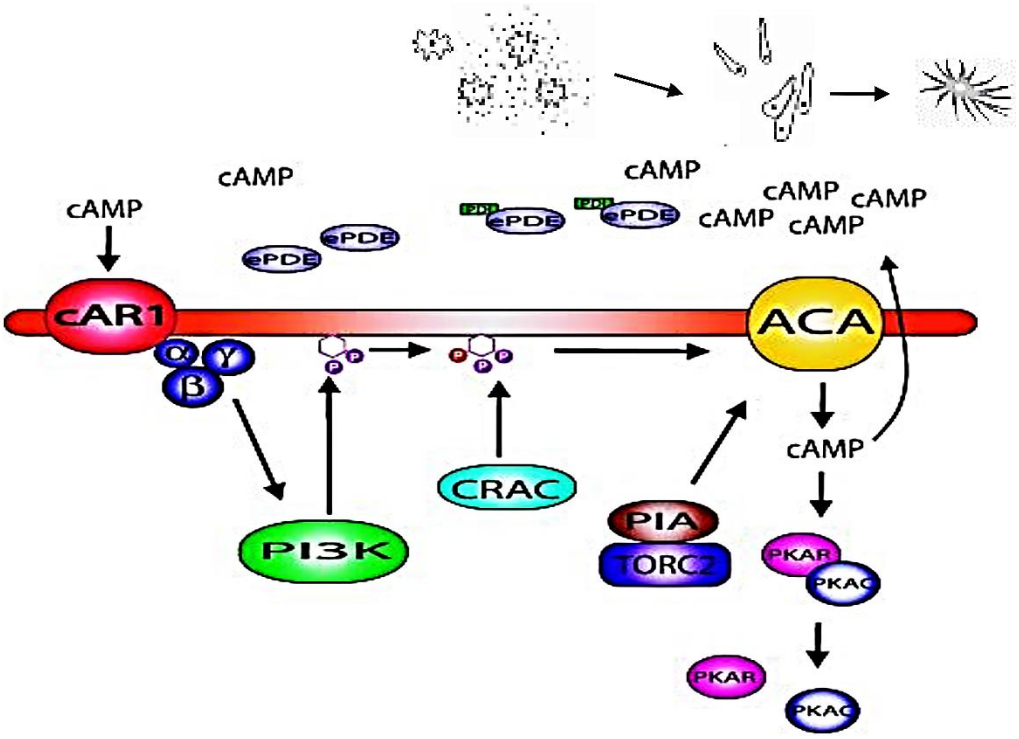
Starvation is an environmental element essential to triggering cell differentiation and a series of morphogenesis in *Dictyostelium*, but it is not enough for the initiation of development. The signals guided by starvation must be integrated into specific events coupled with the transition from growth to development in *Dictyostelium*. During aggregation the cells begin to differentiate into several types with different signaling and chemotactic properties.

2.4.1. The cAMP signaling pathway

Dictyostelium morphogenesis starts with the chemotactic aggregation of starving individual cells. The regulation of this crucial stage of

development revolves around the production, secretion, and inactivation of cAMP. The activation of key components of the cAMP signaling system, such as the major cAMP receptor (cAR1) and the aggregation-stage adenylyl cyclase A (ACA), is one of the earliest responses to starvation in *Dictyostelium*. The cAMP signal relay system employed during aggregation is essential for the development of *Dictyostelium* (Scheme 5). When nutrients are depleted, the cells stop growing and activate starvation responses by secreting a glycoprotein called conditioned medium factor (CMF). Secreted CMF activates cAMP signaling (Mann and Firtel, 1989; Gomer *et al.*, 1991; Yuen *et al.*, 1995). Certain starved cells secrete cAMP, which stimulates neighboring cells to migrate toward cAMP in a head to tail fashion until an aggregate is formed and propagates the cAMP signal to neighboring cells. cAMP gradient is established by the exquisite regulation of the synthesis and degradation of cAMP. cAR1 recognizes secreted cAMP and induces the production of additional cAMP by activating ACA (Van Haastert, 1995). The adaptation process undergoes after stimulation. The transient refractory period the cAMP signaling system is responsible for the outward propagation of cAMP waves because cells which have just relayed the signal are refractory to further stimulation by cAMP. Binding of cAMP to the receptor causes chemotactic movement in the direction of higher cAMP concentration. In addition, *Dictyostelium* cells degrade the extracellular cAMP by using an intricate removal system, ePDE, to prevent the loss of directional information and gene expression resulting from saturation of the receptors.

The cAR1 receptor is essential to aggregation A central element in the chemotactic mechanism is the cAMP receptor, cAR1. The cAMP



Scheme 5. The cAMP signaling pathway during aggregation in *Dictyostelium.* cAMP binding to cAR1 leads to the activation of PI3K and the recruitment of the cytoplasmic, PH-domain containing protein, CRAC. CRAC and other cytoplasmic proteins act in concert to allow the G protein-dependent stimulation of ACA (Mahadeo and Parent, 2006). cAR1, cAMP receptor 1; PI3K, phosphatidylinositol 3-kinase; CRAC, cytosolic regulator of adenylyl cyclase; PIA, pianissimo; TORC2, target of rapamycin complex 2; ACA, adenylyl cyclase; PKAC, protein kinase catalytic subunit; PKAR, protein kinase regulatory subunit; ePDE, extracellular phosphodiesterase.

receptor is a seven trans-membrane domain glycoprotein. It is related to receptors in animals, plants and other simple eukaryotes and is coupled to trimeric GTP-binding proteins (Parent and Devreotes, 1996). There are four receptors for extracellular cAMP (cAR1−4) that are sequentially expressed during *Dictyostelium* development. These receptors display different cell type specificities and different affinities for cAMP. cAR1 and cAR3 have high affinity for cAMP, whereas cAR2 and cAR4 have low affinities for cAMP (Louis *et al.*, 1994; Klein *et al.*, 1988; Saxe *et al.*, 1991; Johnson *et al.*, 1991, 1993). The high affinity cAMP receptors cAR1 is the first to be expressed and the main receptor required for aggregation (Klein *et al.*, 1988; Sun *et al.*, 1990; Sun and Devreotes, 1991). The other high affinity cAMP receptor, cAR3 is partially redundant and can mediate most cAR1-dependent signaling (Insall *et al.*, 1994). Deletion of either gene result in cells that cannot aggregate. The low affinity cAMP receptors cAR2 and cAR4 control events during later times in development (Saxe *et al.*, 1993).

The heterotrimeric G proteins coupled to the cAMP receptors are composed of α, β, and γ subunits. In *Dictyostelium*, G protein complexes may contain 1 of 11 α subunits coupled to a single βγ subunit (Lilly *et al.*, 1993; Wu *et al.*, 1995; Zhang *et al.*, 2001). During aggregation, only Gα2 seems to be coupled to cAR1 and cAR3 to mediate all the cAMP-dependent responses (Kumagai *et al.*, 1989; Sun and Devreotes, 1991). cAMP binding to cAR1/cAR3 induces the exchange of GDP for GTP in the Gα2 subunit and the dissociation of Gα2 from Gβγ. Upon cAMP binding to the receptor, the signal is transduced into the cell through heterotrimeric G-protein-dependent and also independent pathways. Activation of adenylyl cyclase, guanylyl cyclase, and PLC, and modulation of the actin and myosin cytoskeletons are

G protein-dependent effects of cAMP, whereas receptor phosphorylation, Ca^{2+} mobilization, and ERK activation are events that are activated independently of G proteins (Kesbeke *et al.*, 1988; Milne and Coukell, 1991; Milne and Devreotes, 1993; Maeda *et al.*, 1996; Milne *et al.*, 1997; Segall *et al.*, 1995). One of the consequences of cAMP binding to the receptor is cAMP synthesis and secretion. The amount of cAMP made by adenylyl cyclase is proportional to the level of the extracellular stimulus, and the amount of extracellular cAMP depends on how much intracellular cAMP was made (Dinauer *et al.*, 1980).

After cAMP binding on the receptor, there is an adaptive process; a constant level of cAMP causes one burst of synthesis and secretion and then halt. There is no loss of cAR1 from the cell surface. As with many sensory processes, the receptors quickly adapt within ~1−2 min. The adapted receptors, which still bind cAMP, no longer activate intracellular signaling. Within minutes, the extracellular cAMP is hydrolyzed by an extracellular phosphodiesterase (ePDE), allowing the receptors to deadapt and prepare to respond to the next cAMP pulse. In *Dictyostelium*, the alternation between activation and adaptation is essential for relaying the directional cAMP signal necessary for chemotaxis (Devreotes and Zigmond, 1988).

The ACA is highly regulated during aggregation Three distinct adenylyl cyclases are expressed throughout the *Dictyostelium* developmental program; ACA, ACB, and ACG. ACA shares homology with the mammalian G protein-coupled adenylyl cyclases containing two sets of six transmembrane segments each followed by a highly conserved catalytic domain. ACA is expressed during early development and provides the

majority of the cAMP that controls gene induction and aggregation. Remarkably, in addition to the catalytic activity of ACA, the localization of enzyme activity may be important for signal relay. Live cell imaging has shown that ACA is enriched at the rear of chemotaxing cells, suggesting that the mechanism of signal relay may involve restriction of activity to the posterior of cells (Kribel *et al.*, 2003). ACA activity is tightly regulated by the action of the Gα2βγ heterotrimeric complex and two cytoplasmic proteins, cytosolic regulator of adenylyl cyclase (CRAC) and pianissimo. CRAC is a 78 kDa protein containing a N-terminal Pleckstrin Homology (PH) domain that mediates recruitment to the plasma membrane of the leading edge of chemotactic cells on chemoattractant stimulation (Insall *et al.*, 1994; Lilly and Devreotes, 1994, 1995) and it acts as an adaptor between the βγ− complex and ACA (Parent et al., 1998). Pianissimo is also essential for the activation of ACA. The *Pia* gene encodes the *Dictyostelium* homolog of Rictor, a key member of the TORC2 (target of rapamycin complex 2) in mammalian and *Drosophila* (Chen *et al.*, 1997; Sarbassov *et al.*, 2004). It may be required to mediate the binding of CRAC, or act after CRAC has bound to the membrane (Parent *et al.*, 1998). Cells lacking CRAC or Pianissimo are unable to activate adenylyl cyclase and do not aggregate when starved.

ACB is required at the culmination stage, providing much of the cAMP to drive terminal differentiation. Adenylyl cyclase G (ACG) is a unique adenylyl cyclase as it resembles membrane bound guanylyl cyclase in topology (Pitt *et al.*, 1992). ACG contain a large extracellular loop connected to a transmembrane region followed by a single cytoplasmic catalytic loop. The regulation of ACG occurs through an intramolecular switch in the

extracellular loop in response to elevated osmolality in the fruiting body (Saran and Schaap, 2004). ACG is only expressed at the terminal stages of spore formation and its activity helps to maintain spores in a dormant state (Cotter *et al.*, 1999; van Es *et al.*, 1996).

The PKA is essential for gene induction and regulates events in the cytoplasm cAMP-dependent protein kinase A (PKA) plays a critical role during early stage of development and at all later stages. Protein kinase A is a central mediator of development that regulates the levels of expression of genes that respond to cAMP signaling. (Harwood *et al.*, 1992). The PKA of *Dictyostelium* is a dimmer consisting of one regulatory (PKA-R) and one catalytic subunit (PKA-C), rather than the tetramer of higher organisms (de Gunzburg *et al.*, 1986; Anjard *et al.*, 1993). The mRNA for the regulatory subunit of the PKA is present at low levels during growth and the levels increase 10−20 fold during the first 3 hours of development. It continues during the entire course of development (Mutzel *et al.*, 1987). The expression of *pkaC* encoding catalytic subunit of PKA is activated at the onset of development following starvation. Increased *pkaC* expression parallels the activity of PKA and triggers the expression of ACA and cAR1 (Mutzel *et al.*, 1987; Schulkes and Schaap, 1995; Wu *et al.*, 1995; Mann *et al.*, 1997). Overexpression of PKA-C (Anjard *et al.*, 1992; Mann *et al.*, 1992) or the disruption of PKA-R, releasing the catalytic subunit from inhibition, induces rapid development (Abe and Yanagisawa, 1983; Simon *et al.*, 1992). While the disruption of PKA-C leads to developmental failure, aggregateless phenotype, without affecting growth (Mann and Firtel, 1991). Although the PKA activity is present in growing cells, it is not crucial for growth since

PKA-C-null mutants are viable. Thus, the fundamental role of PKA is controlling the earlier events to initiate development.

PKA mediates changes in gene expression responding to cAMP signaling. Several of the genes involved in chemotaxis such as *acaA* (adenylyl cyclase), *pdiA* (the phosphodiesterase inhibitor), and *carA* (the major cAMP receptor in early development), are not transcribed at all in the absence of the PKA catalytic subunit (Mann *et al.*, 1997; Wu *et al.*, 1995). Recent evidence suggests that the role of PKA is achieved by a series of sensor histidine kinases that integrate with the cAMP signaling events (Loomis, 1998; Thomason *et al.*, 1998).

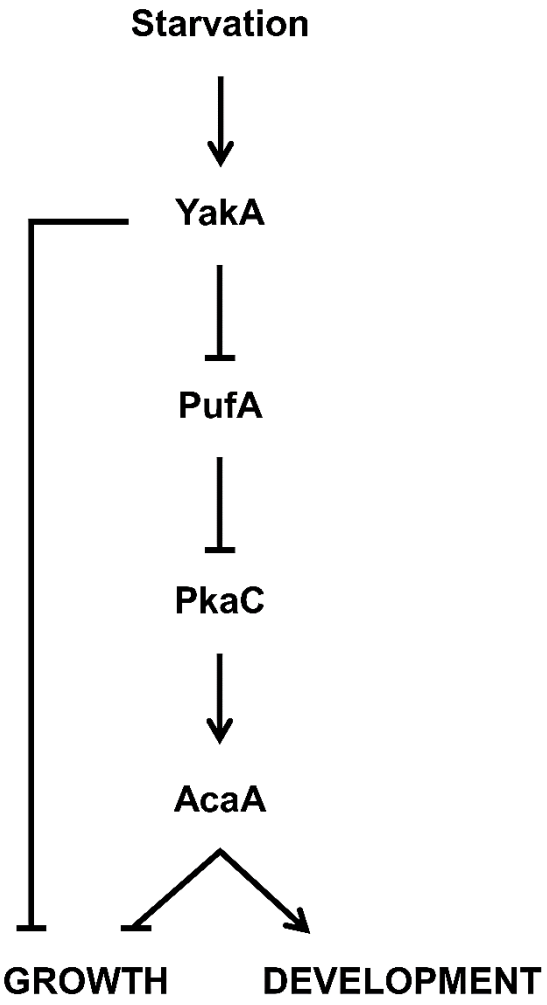
2.4.2. The YakA signaling pathway

The YakA signaling pathway comprises YakA, PufA, and PKA and is activated early when cells are starved. YakA is a member of the dual-specificity tyrosine-related kinase (DYRK) family of serine/threonine kinases and a homologue of yeast Yak1p growth-regulating protein kinase (Souza *et al.*, 1998). The expression of *yakA* is required for turning off growth-phase genes and for induction of differentiation-associated genes. During growth, *yakA* mRNA accumulates and reaches a maximum level at the time of starvation. YakA induces developmental processes such as growth inhibition, downregulates the expression of genes encoding vegetative functions, and upregulates the expression of PKA-C, ACA, and cAR1 (Souza *et al.*, 1998; 1999). *yakA* null cells divide and multiply more rapidly compared to parental cells, reducing their size. PKA-cat mRNA appears normal in *yakA* null cells, but the enzyme activity of PKA does not exhibit the characteristic increase after 5 h of starvation. PKA-dependent

gens are also not expressed in *yakA* null cells. Importantly, *yakA* null cells cannot turn off vegetative genes. The yeast Yak1p is capable of mediating transition from growth to development in *Dictyostelium*, and vice versa, indicating that YakA shares many functions with the yeast Yak1p (Souza *et al.*, 1998).

Other component relating to YakA signaling is PufA. PufA is identified from a mutant which can reverse the aggregateless phenotype of *yakA* null cells to normal. PufA is a member of the Puf (pumilio/FBP) family of proteins, which functions in the translational control of key regulators for anterior-posterior patterning in *Caenorhabditis elegans* and *Drosophila melanogaster* (Forbes and Lehmann, 1998; Wharton *et al.*, 1998; Zamore *et al.*, 1999; Zhang *et al.*, 1997). PufA proteins are sequence-specific RNA binding proteins that bind the 3′ end of mRNA encoding developmentally key regulators. The pumilio protein of *Drosophila* binds to the 3′ end of the hunchback protein and, together with the nanos protein, inhibits the translations of the hunchback protein in the posterior region of oocytes. The RNA sequence (Nanos response elements, NREs) to which pumilio binds have been defined. PKA is likely candidates for regulation by PufA, because *Dictyostelium* PKA mRNA has sequence related to the NRE control elements of the *Drosophila* hunchback protein (Souza *et al.*, 1999).

The YakA signaling regulates the initiation of development by modulating the expression of *yakA* and *pufA*. *pufA* mRNA is present during growth and disappeared by 8 h of starvation. In *yakA* null cells, *pufA* mRNA levels are retained high even after2 h of starvation. Further, the inactivation of *pufA* shows decreased *pufA* expression and allows *yakA* null cells to differentiate, thus indicating that YakA is required for the loss of *pufA* mRNA



Scheme 6. The YakA signaling pathway during aggregation in *Dictyostelium*. Environmental stress signal stimulates the transcriptional expression of YakA. Expressed YakA positively regulates PKA by inhibiting PufA which is proposed to be a direct inhibitor of translation and inhibits the expression of growth-specific genes for the initiation of development of *Dictyostelium* (Taminato *et al*., 2002).

at the onset of development. That is, YakA represses the transcription of *pufA* and thus allows the translation of PKA mRNA to induce development after starvation (Scheme 6). However, genetic perturbations of the signaling events in YakA signaling have not be identified.

3. Aims of this study

GSH is the most prevalent intracellular non-protein thiol compound and performs diverse cellular functions. GSH is essential for survival and differentiation depend on at least some of the multifunctional properties of it. Recently, the critical roles of GSH are reported during growth and development. During growth, the null-mutant of *gcsA* encoding γ-glutamylcysteine synthetase (*gcsA*$^{-}$) shows growth inhibition by methylglyoxal accumulation (Choi *et al.*, 2008) in *Dictyostelium discoideum*. During *Dictyostelium* development, the developmental status is determined by the concentration of intracellular GSH (Kim *et al.*, 2005; Choi *et al.*, 2006). And the expression of *gcsA* is regulated during development, especially it increases during aggregation, indicating that intracellular GSH has role during development of *Dictyostelium*. The evidences for the importance of GSH in early embryonic development are also found in animals and plants. Although GSH has been reported to have a vital role in regulating development, the understanding on the precise action mechanism of GSH is unknown. The elucidation of in what state of development GSH presents a key role and how GSH regulate development will explain fundamental principles of development. The properties of signaling systems which are required for the induction of development in *Dictyostelium* is

remarkably conserved in other higher eukaryotic cells. Thus, the results shown in this study will provide further understanding of general mechanisms of development, in particular the transition from growth to development, not only in *Dictyostelium*, but also in higher organisms.

II. MATERIALS AND METHODS

1. Strains and culture conditions

1.1. *Dictyostelium* strains and culture conditions

1.1.1. *Dictyostelium* strains

Dictyostelium discoideum KAx3 strain was used as a wild-type strain. All mutant strains used in this work were derived from KAx3 cells. γ-Glutamylcysteine synthetase disruption strain (*gcsA*$^{-}$ cells) (Kim *et al.*, 2005), YakA disruption strain (*yakA*$^{-}$ cells), cAMP receptor (cAR1)-expressing strains (cAR1^{OE}/KAx3 and cAR1^{OE}/*gcsA*$^{-}$ cells) and YakA-expressing strains (YakA^{OE}/KAx3 and YakA^{OE}/*gcsA*$^{-}$ cells) were used in this study. The *Dictyostelium* strains used in this study were summarized in Table 1.

1.1.2. Culture condition

Wild-type strain KAx3 and KAx3 mutant cells were grown axenically with shaking at 22 °C in HL5-liquid medium (1.4% thiotone E peptone, 1.4% glucose, 0.7% yeast extract, 3.5 mM $Na_2HPO_4 \cdot 7H_2O$, 4.6 mM KH_2PO4, pH 6.5) containing 100 μg/ml of streptomycin (Duchefa) and 100 units/ml of penicillin (Duchefa) (Cocucci and Sussman, 1970; Soll *et al.*, 1976). To maintain cAR1^{OE}/KAx3, cAR1^{OE}/*gcsA*$^{-}$, YakA^{OE}/KAx3, and YakA^{OE}/*gcsA*$^{-}$ cells, 20 μg/ml of G418 (Duchefa) was supplemented in HL5 medium and to maintain *gcsA*$^{-}$ and *yakA*$^{-}$ cells, 10 μg/ml of blasticidin (ICN) was supplemented in culture medium. For long-term storage of the cells, cell stocks were prepared with 5% DMSO (Sigma-Aldrich)-HL5 and stored at –70 °C. For routinely renewal of cell strain, a frozen stock cell was thawed and suspended in HL5 medium on culture dish plate and used for

experiments by monthly intervals. Every cell culture was harvested during exponential growth.

1.2. Bacterial strains and culture conditions

1.2.1. *Escherichia coli* strains for gene cloning

E. coli DH5α was used for DNA manipulation. DH5α strains were grown at 37 °C on Luria-Bertani (LB, 1% tryptone, 0.5% yeast extract, 1% NaCl) medium with 1.5% agar, where required with the following antibiotics at final concentrations: 50 μg/ml ampicillin (Sigma-Aldrich) and chemicals at final concentrations: 20 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal purchased from Duchefa).

1.2.2. *Klebsiella pneumoniae* strain as a food source of *Dictyostelium*

K. pneumoniae were grown on SM broth (1% glucose, 1% bacto peptone, 1% yeast extract, 4 mM $MgSO_4 \cdot 7H_2O$, 14 mM KH_2PO_4, 5.7 mM K_2HPO_4, prepared as described by Sussman, 1987) with 2% agar. Plates were incubated overnight at 37 °C and stored at 4 °C after sufficient colony growth. Loopful of bacteria harvested from these plates and inoculated for the two-membered *Dictyostelium* cultures. The bacterial strains used in this study were summarized in Table 1.

2. Depletion of GSH

To deplete completely intracellular GSH in *gcsA*$^{-}$ cells, cells were grown exponentially in HL5 media with 1 mM GSH (Duchefa) and were then reinoculated at a density of 2×10^5 cells/ml in media containing 0.5 mM

Table 1. Bacterial and *Dictyostelium discoideum* strains used in this study

Strains	Genotypes	References or sources
Bacterial strains		
E. coli DH5α	F-*ΔlacU169(ϕ80lacZΔM15)endA1 rec1hsdR17 deoR supE44 thi-1 λ-gyrA96 relA1*	Hanahan, 1983
K. pneumoniae		Microbial resources center, 1997
***Dictyostelium discoideum* strains**		
KAx3	Axenic wild-type strain	Firtel, 1997
gcsA^{-}	KAx3:[GCS–Bsr], bs^{r} *gcsA*-disrupted KAx3	Kim, 2005
yakA^{-}	KAx3:[YakA–Bsr], bs^{r} *yakA*-disrupted KAx3	Devreotes, 2001
cAR1^{OE}/KAx3	KAx3:[EXP4(+)–cAR1], neo^{r} cAR1-expressing KAx3	This study
cAR1^{OE}/*gcsA*$^{-}$	*gcsA*$^{-}$:[Exp4(+)–cAR1], neo^{r} cAR1-expressing *gcsA*$^{-}$	This study
YakA^{OE}/KAx3	KAx3:[pTX FLAG–YakA], neo^{r} YakA-expressing KAx3	This study
YakA^{OE}/*gcsA*$^{-}$	*gcsA*$^{-}$:[pTX FLAG–YakA], neo^{r} YakA-expressing *gcsA*$^{-}$	This study

GSH. The exponentially growing cells were inoculated in the same manner in a medium containing 0.2 mM GSH. *gcsA*$^{-}$ cells cultured with 0.2 mM GSH were transferred to fresh HL5 media without GSH and were incubated for 24 h. The experimental scheme is shown in diagram (Fig. 1).

3. Development of *Dictyostelium discoideum*

3.1. Development on non-nutrient agar plates

Development of *Dictyostelium* was induced by removing nutrients. Exponentially growing cells at a density of 2–5 × 10^6 cells/ml were washed twice with non-nutrient KK2 buffer (16.5 mM KH_2PO_4, 3.8 mM K_2HPO_4, pH6.2) and the buffer was removed by centrifugation at 500 g for 5 min. And then the cells were suspended with KK2 buffer and plated at a density of 2 × 10^6 cells/cm^2 on a nitrocellulose filter or on 1.5% KK2 agar plates. Cells plated on agar plates were incubated at 22 °C for desired time. For the development of *gcsA*$^{-}$ cells, exogenous 1 mM GSH was supplemented to KK2 buffer before subjecting on plates.

3.2. Development in non-nutrient buffer

To induce development in suspension, cells were washed with KK2 buffer twice and the buffer was removed by centrifugation at 500 g for 5 min. And then washed cells were suspended in non-nutrient KK2 buffer at a density of 1 × 10^7 cells/ml and shaken at 150 rpm at 22 °C. For the development of GSH-depleted *gcsA*$^{-}$ cells, 1 mM GSH (Duchefa) was added to development buffer. After 2 h of development, cAMP stimulation was performed by adding of 30 nM cAMP (Sigma-Aldrich) every 6 min. 30 nM

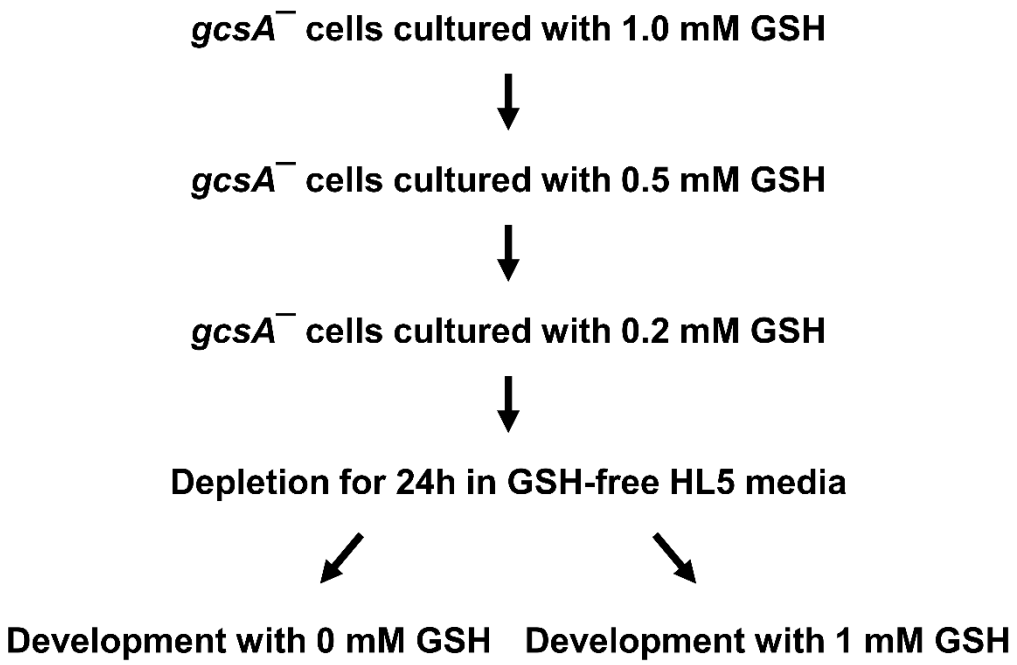


Fig. 1. Experimental scheme for the complete depletion of intracellular GSH and for the development of GSH-depleted *gcsA*¯ cells. Intracellular GSH was depleted from the cells gradually and GSH-depleted *gcsA*¯ cells were subjected on non-nutrient KK2 agar or in non-nutrient KK2 buffer.

cAMP was supplemented for 4 h and after then 300 μM cAMP was added every 2 h for 4 h (Galardi-Castilla *et al.*, 2008). The experimental scheme for the exogenously added cAMP pulses is presented in diagram (Fig. 2).

To examine the effect of other thiol compounds or antioxidant on the development of *gcsA*$^{-}$ cells, 1 mM oxidized glutathione (GSSG purchased from Sigma-Aldrich), 1 mM γ-glutamyl cysteine (γ-GC purchased from Sigma-Aldrich), 1 mM dithiothreitol (DTT purchased from Duchefa), 1 mM *N*-acetylcysteine (NAC purchased from Sigma-Aldrich), and 1 mM ascorbic acid (Sigma-Aldrich) were supplemented to non-nutrient KK2 buffer.

4. Transformation of *Dictyostelium discoideum*

The transformation of *Dictyostelium* cells was performed according to the protocol (Schlatterer *et al.*, 1992) with some modification (Pang *et al.*, 1999). *Dictyostelium* cells were grown axenically in suspension culture to a density of 2–3 × 10^6 cells/ml. Cells were washed twice with an equal volume of ice-cold H-50 buffer (20 mM HEPES, 50 mM KCl, 10 mM NaCl, 1 mM $MgSO_4$, 5 mM $NaHCO_3$, 1 mM NaH_2PO_4, pH 7.0, autoclaved and stored cold or frozen). The washing buffer was removed by centrifugation at 500 × g for 5 min at 4 °C. After washing, the cells were resuspended in H-50 buffer at a density of 5 × 10^6 cells/ml. For electroporation, 1–10 μg of plasmid DNA was added to 100 μl of suspended cells in H-50 buffer and the cell-DNA mixture was transferred to a pre-chilled electroporation cuvette (0.1 cm electrode gap, Bio-Rad). The cells were sparked with electricity at 0.85 kV / 25 μF twice with about 5 sec between pulses. After electroporation, the cells were incubated in the cuvette on ice for 5 min and suspend into a cell culture

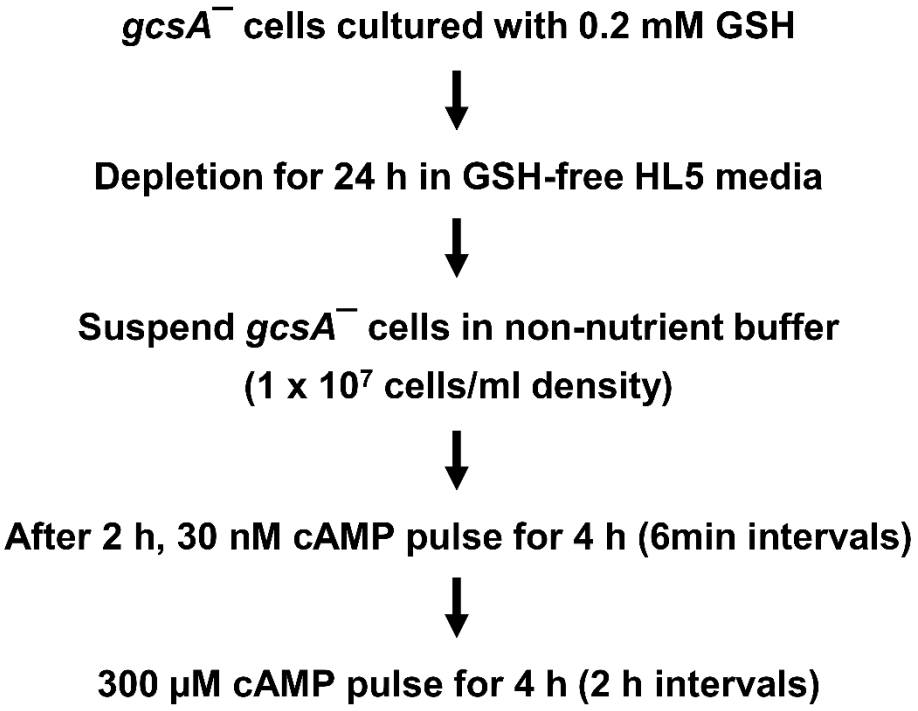


Fig. 2. Experimental scheme for suspension development of GSH-depleted *gcsA*$^{-}$ cells with cAMP pulses. Exogenous cAMP was supplemented as the same condition of internal cAMP secretion at 6 m intervals and at nanomolar levels.

dish with 10 ml of HL5 medium. After 18–24 h incubation at 22 °C, the medium was replaced by the selection medium containing appropriate antibiotics (20 μg/ml of G418 purchased from Duchefa or 10 μg/ml blasticidin S purchased from ICN).

5. Genetic manipulation methods

General techniques for isolation and manipulation of DNA in *E. coli* were as previously described (Sambrook and Gething, 1989). pGEM-T easy vector (Promega) was used for cloning of PCR product. Integrating expression vector Exp4(+) (Dynes *et al.*, 1994) and extrachromosomal expression vector pTX-FLAG (Levi *et al.*, 2000) were used for introducing appropriate genes into *Dictyostelium*. The constructs and plasmids used in this study were summarized in Table 2.

5.1. Isolation and subcloning of *carA* from *Dictyostelium discoideum*

Full-length of *carA* (1.3 kb) was amplified by polymerase chain reaction (PCR) using genomic (g)DNA as the template. The PCR-primer sequences were as follows: forward 5′-GGATCCATGGGTCTTTTAGATGGAAATCC A-3′ and reverse 5′- CTCGAGATCAATTATTTCCTTGACCATTT-3′. The amplified product was cloned into pGEM-Teasy cloning vector (Promega), yielding pGEM–Teasy–cAR1. The construct was digested with *Bam*HI and *Xho*I and a construct for constitutive expression of cAR1 was generated by cloning the full-length gDNA *carA* amplicon into the Exp4(+) vector containing a constitutively active *Act15* promoter. The constructs were

Table 2. Plasmids and constructs used in this study

Plasmids	Descriptions	References or sources
pGEM-Teasy	PCR cloning vector	Promega
Exp4(+)	Expression vector for *Dictyostelium*	Firtel, 1997
pTX–FLAG	FLAG-tagged protein expression vector	Egelhoff, 2000
pGEM–Teasy–cAR1	pGEM–T easy vector containing *carA* ORF	This study
Exp4(+)–cAR1	Exp4(+) vector fused with *carA* ORF in frame	This study
pGEM–Teasy–YakAF1	pGEM–T easy vector containing fragmented *yakA* ORF (1-2621)	This study
pGEM–Teasy–YakAF2	pGEM–T easy vector containing fragmented *yakA* ORF (2622-4377)	This study
pGEM–Teasy–YakA	pGEM–T easy vector containing full-length of *yakA* ORF	This study
pTX-FLAG–YakA	pTX–FLAG vector fused with *yakA* ORF in frame	This study

introduced into KAx3 or *gcsA*$^{-}$ cells using electroporation (Pang *et al.*, 1999), and transformants were selected and maintained in medium containing 10 μg/ml of G418 (Duchefa).

5.2. Isolation and subcloning of *yakA* from *Dictyostelium discoideum*

Full-length of *yakA* (4.3 kb) amplified into two fragments by polymerase chain reaction (PCR) using complementary (c)DNA as the template: first fragment (YakAF1) with *Sac*I and *Bam*HI restriction enzyme site, second fragment (YakAF2) with *Bam*HI and *Xho*I restriction enzyme site. These two fragments were ligated and cloned into pGEM-Teasy cloning vector (Promega), yielding pGEM–Teasy–YakA. The primer sequences of *yakA* fragment 1 (1 to 2621) for PCR were as follows: forward 5′-CAATAGAGCTCATGGGCAGTACTACACAAATGAGC-3′ and reverse 5′-GTGGATCCATTCCCTCTGAACTTG-3′. The primer sequences of *yakA* fragment 2 (2622 to 4377) were as follows: forward 5′-CAAGTTCAGAGG GAATGGATCCAC-3′ and reverse: 5′-GTATATATTTTCTCGAGTTATGTC TCTCTATATGAACCAATAACAACC-3′. The construct was digested with *Sac*I and *Xho*I and a construct for constitutive expression of cAR1 was generated by cloning the full-length cDNA *yakA* amplicon into the pTX-FLAG vector containing a constitutively active *Act15* promoter. The constructs were introduced into KAx3 or *gcsA*$^{-}$ cells using electroporation (Pang *et al.*, 1999), and transformants were selected and maintained in medium containing 10 μg/ml of G418 (Duchefa).

5.3. Polymerase chain reaction (PCR)

DNA fragment amplification was performed according to the method recommended by Taq polymerase manufacturer (Promega, Madison, WI) with slight modification. For the reaction, 25 pmol of degenerate oligonucleotide primers, 100 ng of cDNA or gDNA and 0.25 units of Taq polymerase were combined in a final volume of 25 μl with reaction buffer (50mM KCl, 1.2 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.4, 0.01% gelatin) containing 50 μM of each dNTP. The mixture was subjected to 30 cycles of 30 sec denaturation at 94 °C, 30 sec annealing at 55 °C and 1 min extension at 72 °C.

5.4. Real-time reverse transcriptase-polymerase chain reaction (Real-time RT-PCR)

Each RNA sample (50 ng/μl) was reverse-transcribed into cDNA using superscript III Reverse Transcriptase Kit (Promega). Real-time PCR was performed in a 20-μl volume in the well of 96-well reaction plates (Bioplastics). Each PCR assay was performed using SYBR Premix Ex Taq (TaKaRa), and *rnlA* served as an endogenous control. Fluorescence was detected using an Applied Biosystems 7500 real-time PCR system. The reactions for each gene at each time point were performed in triplicate, and cycle threshold values generated from the reactions were averaged. The cycle threshold values of each gene were normalized to the endogenous controls and calibrated to an average expression level for the gene being analyzed (Kim *et al.*, 2011). The primer sequences of *yakA* for real-time RT-PCR were as follows: forward 5′-CACCTTTGATGATGTCACAACCAC-3′ and reverse 5′-ATAGAAGATGCATCACCCATCAATG-3′. The primer

sequences of *rnlA* were as follows: forward 5′-ACTAGGCAGACTATGAG CGCTAAGG-3′ and reverse: 5′-CTGTAGATTGTTGGCTAGAGAAC-3′.

5.5. RNA extraction and Northern blot analysis

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the supplier's recommendations and solubilized in formamide (Sigma-Aldrich). The RNA (20 μg) sample was separated by electrophoresis through a 1% agarose gel containing 0.22 M formaldehyde (Sigma-Aldrich) and then transferred to Hybond-N+ nylon membrane (GE Healthcare). The specific probes were labeled with [α-^{32}P]-dATP (Feinberg and Vogelstein, 1983). The primer sequences of each probes for hybridization were summarized in Table 3. Hybridization was performed using various probes dissolved in Rapid-Hyb buffer (GE Healthcare) according to the manufacturer's instructions. The blots were incubated in Rapid-Hyb buffer without the probe for 1 h and then probe was added for 2 h. The blot was washed twice with SSC buffer (0.1% SDS, 0.3 M NaCl, 30 mM trisodium citrate) for 10 min at 65 °C. The signal was visualized by exposing membrane to X-ray film. All the solutions were treated with diethyl pyrocarbonate (DEPC purchased from Sigma-Aldrich) and autoclaved.

6. Measurement of PKA activity

PKA activity measurements were performed as described previously (Wang and Kuspa, 2002) using the SignaTECT PKA Activity System (Promega). Samples were prepared from cells developed in non-nutrient KK2 buffer with cAMP pulses. Harvested cells by centrifugation at 500 ×

Table 3. List of primer sequences used in Northern blot analysis for the preparation of hybridization probes

Probes	Primer sequences (5′ to 3′)	Position
cprD	F[a]: CAGCCCTCATTGGTACTGAAGAAG R[b]: CGATCCAGTAGTTACCAGATGAGGCC	290 to 1210
carA	F: GAGAACCAGAACCAGAAAGATTTG R: TTTCCTTGACCATTTGTTGAAGTGG	332 to 1172
acaA	F: GGATCCTGCACCTTATTTCAATAG R: CTCGAGATTTGGTTAATGCAGATTGTGGG	2952 to 3499
gpaB	F: ATGGGTATTTGTGCATCATCAATGGAAG R: CAGTTGGAATATAAACTGGTGATGTCATACG	1 to 523
pkaC	F: GAATTCTCAAGGTCACATTAAAATCACTG R: GAATTCGGAGGCTCTTCAACCATTTCTTC	1407 to 1898
pkaR	F: GGATCCATGACAAATAATATATCACATAACC R: CTCGAGTTAAGATTTTTGAGAGGTTAAATTTGG	1 to 984
pufA	F: CACCCTGTAGTTACATTATCATCATCAC R: GGTTGTTGCTGCTGATGACAATGATGACG	1120 to 1620
dscA	F: ATGTCTACCCAAGGTTTAGTTC R: TTATTCCAAAGCGGTAGCAATG	1 to 762
dia2	F: ATGAAACAAATTATTAGATTAATAACTAC R: GTTTGGAATAACTTGATATAATTTTCCAG	1 to 453
gcsA	F: CGATGATGAAAAGAATACAGATC R: TTAACAATAATAATCATCTTTATC	1200 to 1881
rnlA	F: GGCGGAACCCGTAACTGTTGCAAAAG R: CACAATTATACGGAACATTTTACTACC	695 to 1216

[a] F, forward primer; [b] R, reverse primer.

g for 5 min at 4 ℃ were sonicated and supernatant were separated by centrifugation at 12,000 rpm for 15 min at 4 ℃. Cell extracts containing 10 μg of protein were prepared as specified by the manufacturer and were used in reactions with 10 μM cAMP and in the presence or absence of 20 μM of the PKA-specific inhibitor PKI (Mann *et al.*, 1992). PKA activity is defined as the amount of the phosphorylated substrate, kemptide (nmol/min/mg of protein), in the absence of PKI minus the amount of phosphorylated substrate in the presence of PKI.

7. Measurement of glutathione concentration

To determine the concentration of intracellular glutathione, cell extracts were reacted with monobromobimane (mBBr) to form derivates and then analyzed using a modification of method described by Newton and Fahey (1995). Cells developed in non-nutrient KK2 buffer in suspension and were harvested by centrifugation at 500 × g for 5 min at 4 ℃. Prepared cells were extracted with 50% aqueous acetonitrile (Sigma-Aldrich) containing 50 mM Hepes (pH 8.0), 2 mM EDTA, and 2 mM mBBr (Sigma-Aldrich). After incubation at 60℃ for 15 min, the samples were acidified with 5 μl of 5 N methanesulfonic acid (Sigma-Aldrich). Cell debris was removed from the crude extract by centrifugation at 12,000 rpm for 15min, and the resulting supernatant was analyzed using HPLC. Control samples were treated with 5 mM *N*-ethylmaleimide (NEM purchased from Sigma-Aldrich) and incubated for 10 min before derivatization to prevent labeling of thiol group from with mBBr. The concentration of total GSH is determined using 2 mM dithiothretol (DTT), which reduces GSSG to GSH. Samples (10 μl) were

passed through a ZORBAX SB-C18 column. HPLC was performed using a Waters system equipped with a Hewlett-Packard 1050 series fluorescence detector. The mBBr-derived thiol compounds were detected using excitation and emission at 370 and 480 nm, respectively. The mobile phase consisted of buffer A (methanol, HPLC grade from Sigma-Aldrich) and buffer B (0.1% trifluoroacetic acid from Sigma-Aldrich). The proportion of buffer A in the continuous gradients was as follows; 15% at 0–2 min, 25% at 30 min, 100% at 34 min, 15% at 37 min, and 15% at 40 min. If necessary, samples were co-injected with GSH (Duchefa) standards.

III. RESULTS

1. The roles of GSH in development of *Dictyostelium discoideum*

1.1. Complete depletion of GSH in *Dictyostelium*

Previously, it is demonstrated that GSH is essential for the normal development of *Dictyostelium* (Kim *et al.*, 2005). *gcsA*$^{-}$ cells exhibit different developmental morphologies as GSH concentration which is exogenously added to culture media before development. *gcsA*$^{-}$ cells are arrested at mound stage when pre-cultured with 0.2 mM GSH and at culmination step when pre-cultured with more than 0.5 mM GSH. Prespore-specific genes and spore-specific genes are not expressed in *gcsA*$^{-}$ cells. In the present study, to address GSH functions in what developmental stage and how regulates the development of *Dictyostelium*, developmental morphology was observed when intracellular GSH was depleted completely. For the complete depletion of GSH in *gcsA*$^{-}$ cells, cells grown in HL5 media with 1 mM GSH were re-inoculated to media with 0.5 mM GSH, and then to 0.2 mM GSH gradually. Finally, cells cultured with 0.2mM GSH transferred to fresh HL5 media with no GSH and incubated for 24h as mentioned in materials and methods. The depletion of GSH was confirmed by measuring the concentration of intracellular GSH using HPLC connected to a fluorescence detector. The intracellular GSH level was not detected in *gcsA*$^{-}$ cells with no addition of GSH (Fig. 3). It was recovered to around 60% of KAx3 in *gcsA*$^{-}$ cells when cultured with the exogenous addition of 1 mM GSH. Intracellular GSH was removed efficiently and furthermore, an empirical study of the role of GSH in the regulation of development of *Dictyostelium* was possible by producing the GSH-depleted cells.

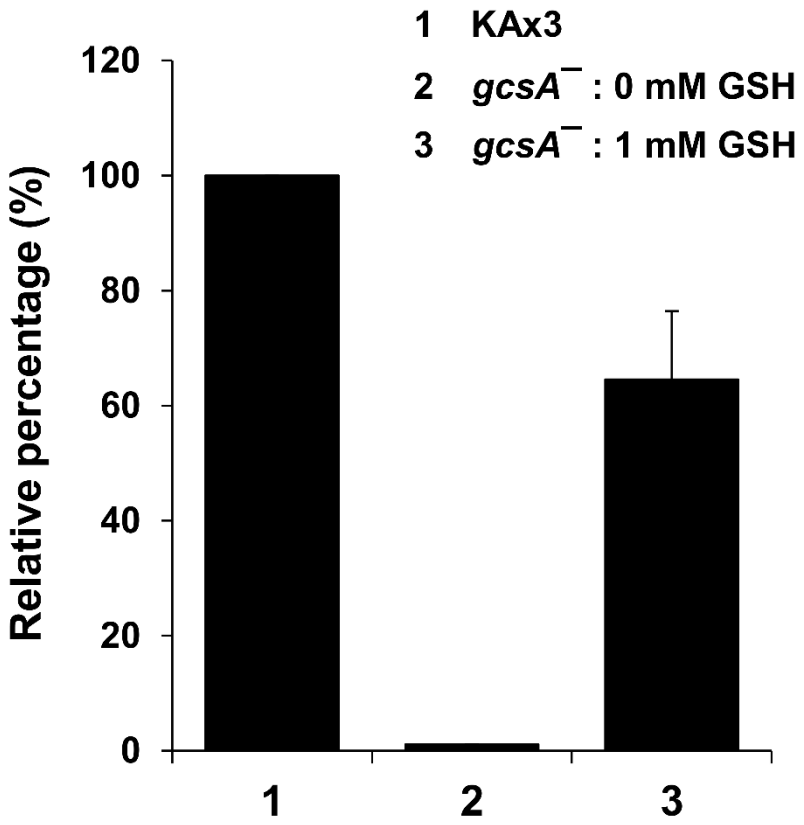


Fig. 3. Complete depletion of intracellular GSH. The concentration of intracellular GSH was measured in exponentially growing KAx3 cells, GSH-depleted *gcsA*¯ cells (*gcsA*¯: 0 mM GSH), and *gcsA*¯ cells which were cultured with 1 mM GSH (*gcsA*¯: 1 mM GSH) using HPLC and fluorescent detector. Intracellular GSH was modified to a mBBr-conjugated form to detect. The concentration of GSH was calculated in relative values compared to that of KAx3 cells. The values represent the mean ± S.E.M. of three independent experiments.

1.2. The roles of GSH in development on agar plates

Developmental morphology of GSH-depleted *gcsA*$^{-}$ cells was observed in the presence of GSH or in the absence of GSH. GSH-depleted *gcsA*$^{-}$ cells did not develop without the addition of GSH when subjected on non-nutrient KK2 agar plates (Fig. 4). However, they formed the final developmental structure, fruiting bodies, with the addition of 1 mM GSH. The efficiency of fruiting body formation and the viability of spore were much lower than those of wild-type KAx3 cells as described by Kim *et al.* (2005). Because the GSH-depleted *gcsA*$^{-}$ cells did not initiate development, the early developmental state, aggregation process, was monitored closely using a phase-contrast microscope. KAx3 cells agglomerated together to form aggregates and formed tipped aggregates at 12 h in response to starvation signal (Fig. 5). *gcsA*$^{-}$ cells did not aggregate without GSH but formed aggregates clearly with distinct stream patterns when 1 mM GSH was exogenously added though the process was slightly delayed compared to KAx3 cells. According to these results, it is clear that GSH regulates development of *Dictyostelium*, particularly the initiation of development.

1.3. The roles of GSH in aggregation processes

There were limits on observing the procedures of formation of aggregates on non-nutrient agar plates. For detailed analysis, cells were induced to develop in suspension, because this is an effective method for observing early developmental processes, especially cell aggregation. KAx3 cells developed and formed aggregates in non-nutrient KK2 buffer (Fig. 6). However, *gcsA*$^{-}$ cells did not form aggregates in the absence of added GSH

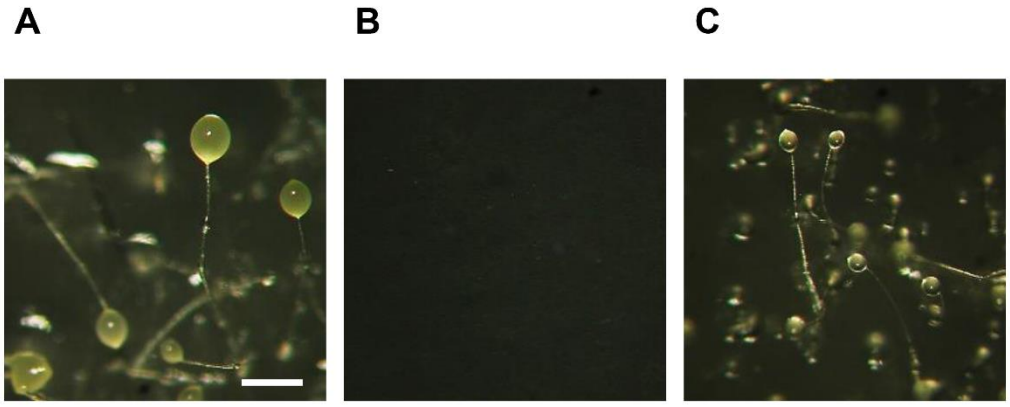


Fig. 4. Developmental morphology of KAx3 and *gcsA*$^{-}$ cells on non-nutrient KK2 agar plates. KAx3 cells and GSH-depleted *gcsA*$^{-}$ cells were allowed to develop on non-nutrient KK2 agar plates with or without the addition of 1 mM GSH under overhead lightening and photographed at 24 h after development. (A) KAx3 cells, (B) *gcsA*$^{-}$ cells without GSH, (C) *gcsA*$^{-}$ cells with 1 mM GSH. The scale bar represents 0.25 mm.

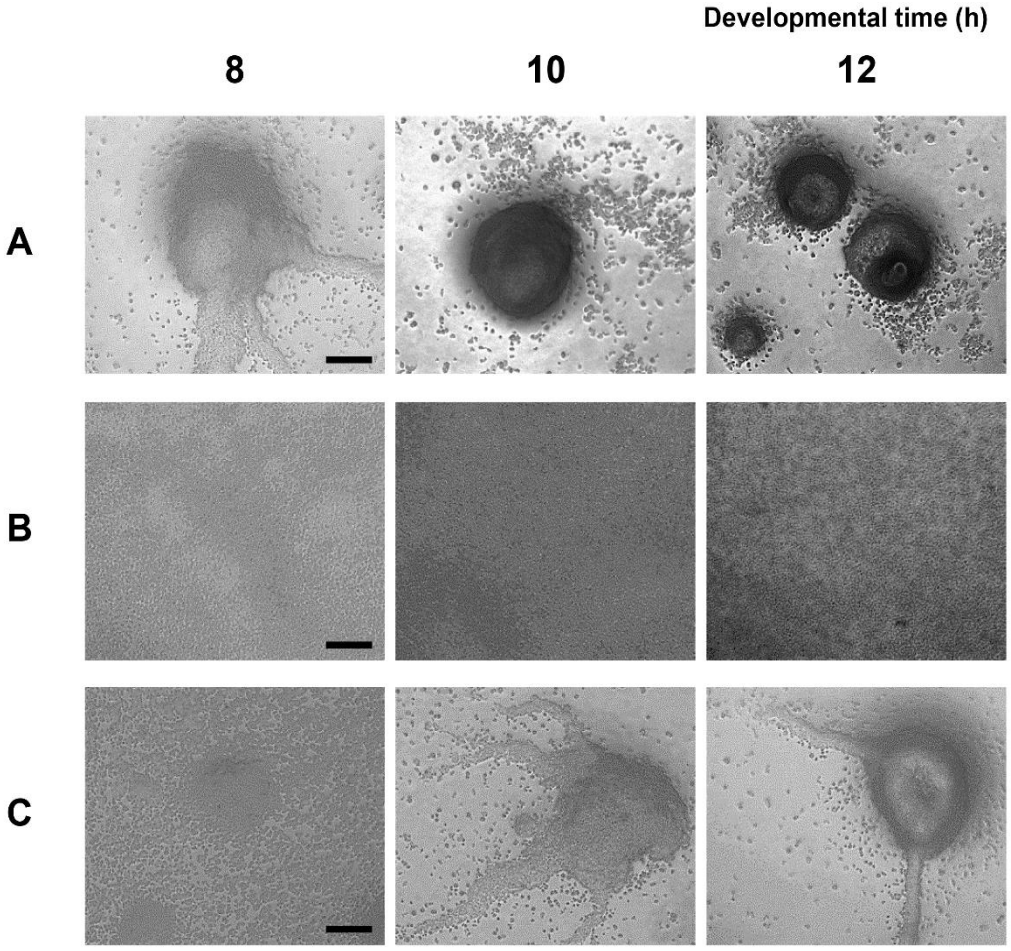


Fig. 5. Developmental morphology of KAx3 and *gcsA*$^{-}$ cells during aggregation on non-nutrient KK2 agar plates. KAx3 and *gcsA*$^{-}$ cells were subjected on KK2 plates with or without the addition of 1 mM GSH under an overhead light. The process of aggregation was observed at the indicated time using a phase-contrast microscope. (A) KAx3 cells, (B*) gcsA*$^{-}$ cells without GSH, (C) *gcsA*$^{-}$ cells with 1 mM GSH. The scale bar represents 0.1 mm.

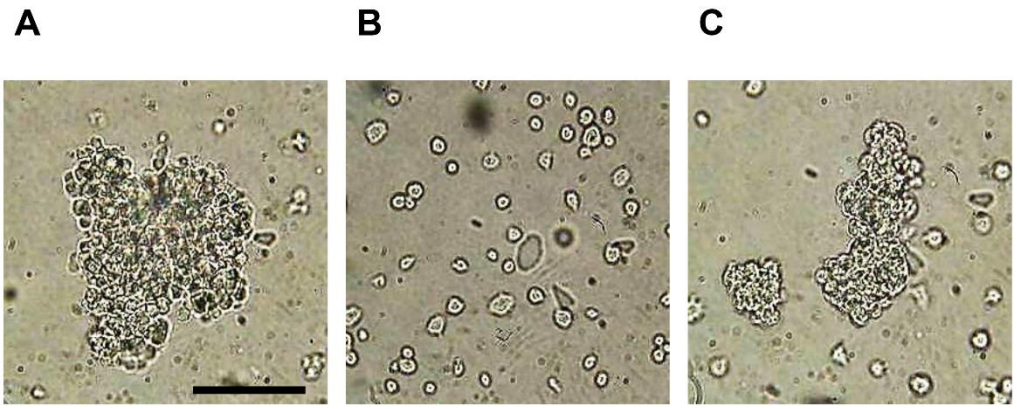


Fig. 6. Developmental morphology of KAx3 and *gcsA*$^{-}$ cells in suspension without cAMP pulses. KAx3 and *gcsA*$^{-}$ cells were allowed to develop in non-nutrient KK2 buffer in the presence of 1 mM GSH or in its absence and photographed at 12 h. (A) KAx3 cells, (B) *gcsA*$^{-}$ cells without GSH, (C) *gcsA*$^{-}$ cells with 1 mM GSH. The scale bar represents 0.05 mm.

and consistently remained as single cells. *gcsA*$^{-}$ cells formed aggregates in the presence of 1 mM GSH, although they were small compared with KAx3 cells. To examine that restoration of the developmental defect of GSH-depleted *gcsA*$^{-}$ cells was due to the supplementation of exogenous GSH, the intracellular GSH level was monitored after treatment of 1 mM GSH in *gcsA*$^{-}$ cells during suspension development. It was approximately 10% of that in KAx3 cells after commencement of starvation (0 h) and increased gradually up to approximately 40% at 4 h after the supplementation of GSH (Fig. 7). The level was not increased further and was sustained. In *gcsA*$^{-}$ cells without the addition of GSH, intracellular GSH was not detected. The relatively low intracellular GSH levels in *gcsA*$^{-}$ cells to the levels in KAx3 cells in the presence of the added 1 mM GSH may explain delayed developmental processes, as shown in Figs. 3, 4, and 5. These results indicate that GSH functions for the developmental initiation in *Dictyostelium*.

1.4. Irreplaceable roles of GSH by other antioxidant molecules

In addition to exogenous GSH, the effect of a precursor of GSH biosynthesis, γ-GC (γ-glutamylcysteine), and oxidized form of glutathione, GSSG, on the developmental morphology of GSH-depleted *gcsA*$^{-}$ cells was examined. *gcsA*$^{-}$ cells aggregates in the presence of both 1 mM γ-GC and GSSG, though the rate of formation of aggregates was significantly slow than that of KAx3 and *gcsA*$^{-}$ cells with the addition of GSH (Fig. 8). These results indicate that not only GSH but also the compounds which could be converted to GSH inside of cells rescue the developmental defects of *gcsA*$^{-}$ cells.

The role of GSH as an important antioxidant via its potent reducing

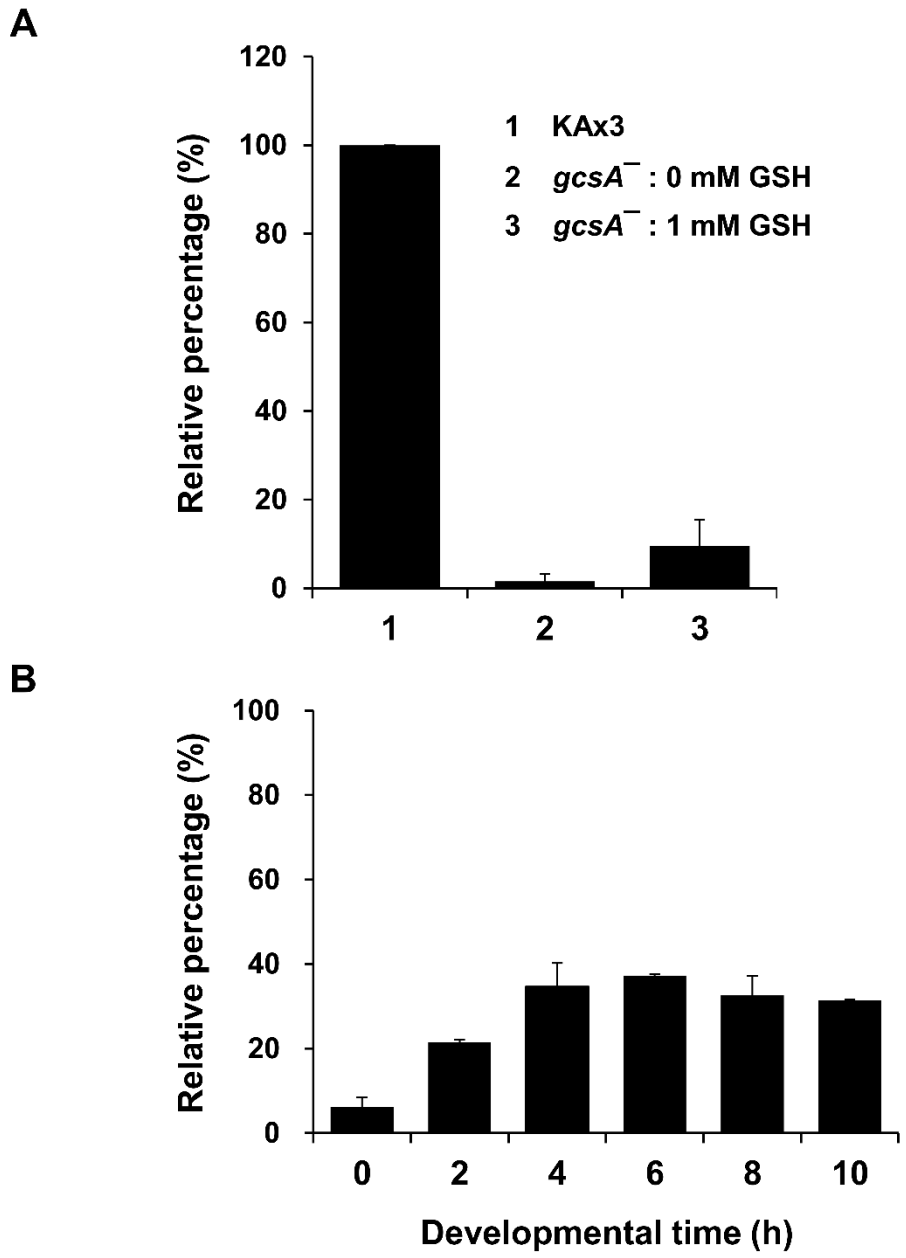


Fig. 7. Intracellular GSH concentration of *gcsA*¯ cells during suspension development. (A) The relative values of the GSH concentration in *gcsA*¯ cells without or with 1 mM GSH compared to that of KAx3 cells at 0 h of development. (B) The relative values of the GSH concentration of *gcsA*¯ cells with 1mM GSH during aggregation compared to that of KAx3 cells. The values represent mean ± S.E.M. of three independent experiments.

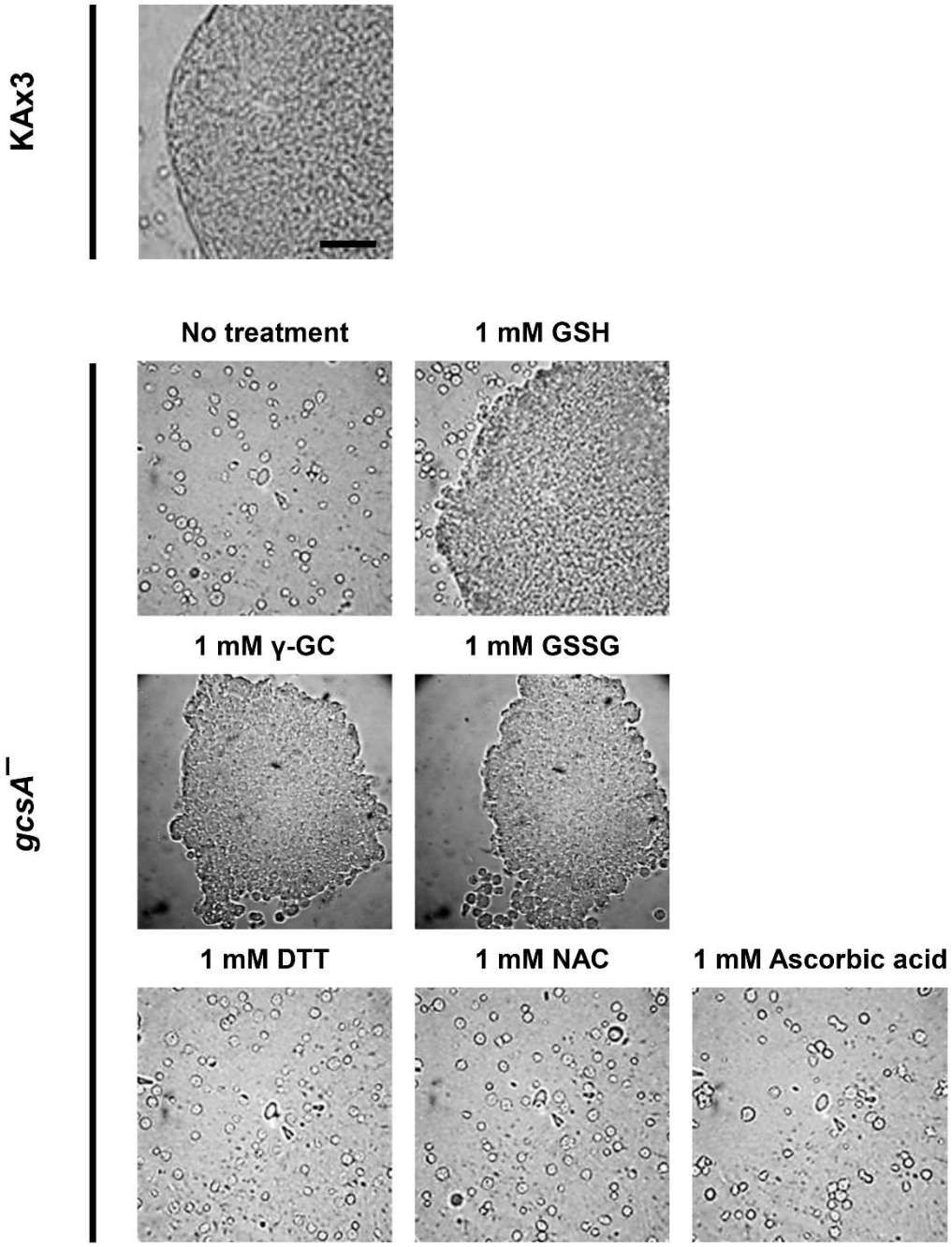

KAx3
No treatment
1 mM GSH
gcsA⁻
1 mM γ-GC
1 mM GSSG
1 mM DTT
1 mM NAC
1 mM Ascorbic acid

Fig. 8. Effect of other exogenous thiols or reducing agents on the development of KAx3 and *gcsA*$^{-}$ cells in suspension. KAx3 and *gcsA*$^{-}$ cells were subjected in non-nutrient KK2 buffer with 1 mM of γ-GC (γ-glutamyl cysteine), GSSG (oxidized glutathione), DTT (dithiothreitol), NAC (*N*-acetylcysteine), and ascorbic acid. The developmental morphology was observed at 12 h after development. The scale bar represents 0.05 mm.

potential is widely known. Depletion of GSH from cells may cause oxidative stress and lead to developmental defects in *Dictyostelium*. This possibility was examined that whether other thiol-containing compounds or reducing compounds rescued the developmental defects of *gcsA*$^{-}$ cells (Fig. 8). It was found that GSH-depleted *gcsA*$^{-}$ cells failed to form aggregates regardless of the concentration of thiol-containing molecule and a general reducing compound, such as dithiothreitol (DTT), *N*-acetylcysteine (NAC), and ascorbic acid. Only GSH induced the GSH-depleted *gcsA*$^{-}$ cells to initiate development. These results suggest that GSH plays indispensable roles independent of its redox properties in the initiation of *Dictyostelium* development.

2. Developmental properties of GSH-depleted *gcsA*$^{-}$ cells

It is known that the transcriptional expression of genes are regulated for developmental initiation in *Dictyostelium*. The expression of vegetative genes, which are most needed to maintain the growth and energy metabolism, decreases. But the expression of developmental genes, which are required for the proper progression of developmental life cycle, increases. To gain more information on the developmental status of *gcsA*$^{-}$ cells, the expression of genes that were needed to be regulated for optimal developmental initiation was monitored. KAx3 and *gcsA*$^{-}$ cells were allowed to develop in suspension and total RNA samples were prepared at various developmental times. The *cprD* expresses a cysteine protenase during growth but not during

development (Souza *et al.*, 1998). Northern blot analysis revealed that the expression level of *cprD* decreased in response to starvation signal and it was hard to detect at 4 h after development in KAx3 cells (Fig. 9). In contrast, the level of *cprD* remained high during development in *gcsA*$^{-}$ cells without the addition of GSH. When 1 mM GSH was added to *gcsA*$^{-}$ cells, the expression pattern of *cprD* was similar to that of KAx3 cells, although it was slightly delayed.

Further, the expression of *dscA* and *dia2* was observed. *dscA* and *dia2* are known as good markers for the transition from growth to development (Maeda, 2005), because their mRNA transcripts accumulate only during development. *dscA* and *dia2* were not expressed in GSH-depleted *gcsA*$^{-}$ cells (Fig. 10). However, the addition of 1 mM exogenous GSH induced the expression of them. The expression of *cprD, dscA*, and *dia2* seems to be modulated by GSH. These results indicate that GSH-depleted *gcsA*$^{-}$ cells are not ready to initiate developmental cycle.

3. The roles of GSH in the regulation of cAMP signaling

3.1. The expression of genes related with the cAMP signaling system in *gcsA*$^{-}$ cells

GSH depletion caused halt of life cycle progression from growth to development in *Dictyostelium*. GSH-depleted *gcsA*$^{-}$ cells did not form aggregates and existed as single cells. The cAMP signaling pathway is one of the earliest events to induce aggregation in multicellular development of *Dictyostelium* (Loomis, 1998). *carA* and *acaA*, encoding cAMP receptor cAR1 and adenylyl cyclase ACA, respectively, are key regulators of cAMP

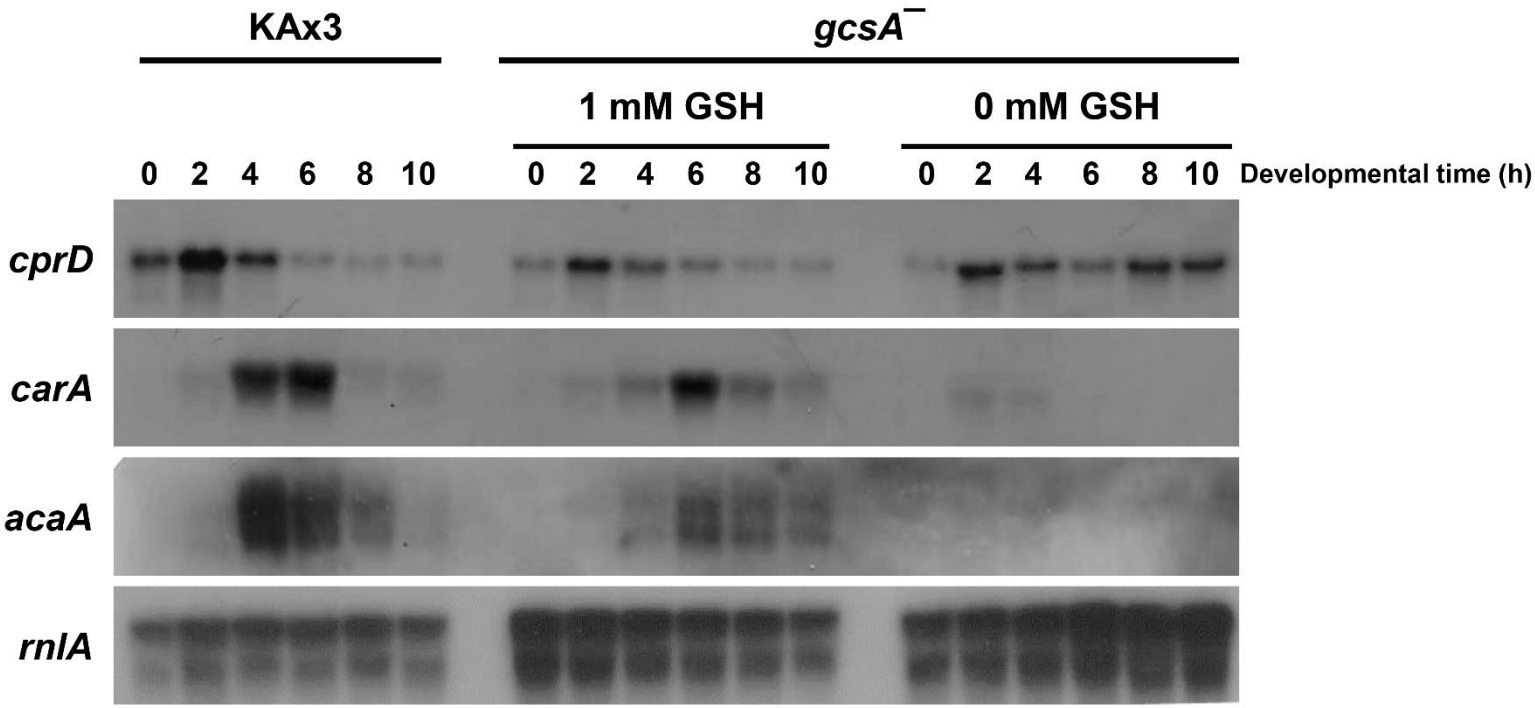


Fig. 9. Expression of early developmental genes in *gcsA*¯ cells during suspension development. KAx3 and *gcsA*¯ cells were allowed to develop in non-nutrient KK2 buffer for 10 h and the expression of genes, which were known to be regulated for the developmental initiation, was analyzed by Northern blotting. *cprD*, encoding vegetative-stage-specific serine proteinase; *carA*, encoding cAMP receptor 1; *acaA*, encoding adenylyl cyclase A; *rnlA*, a loading control.

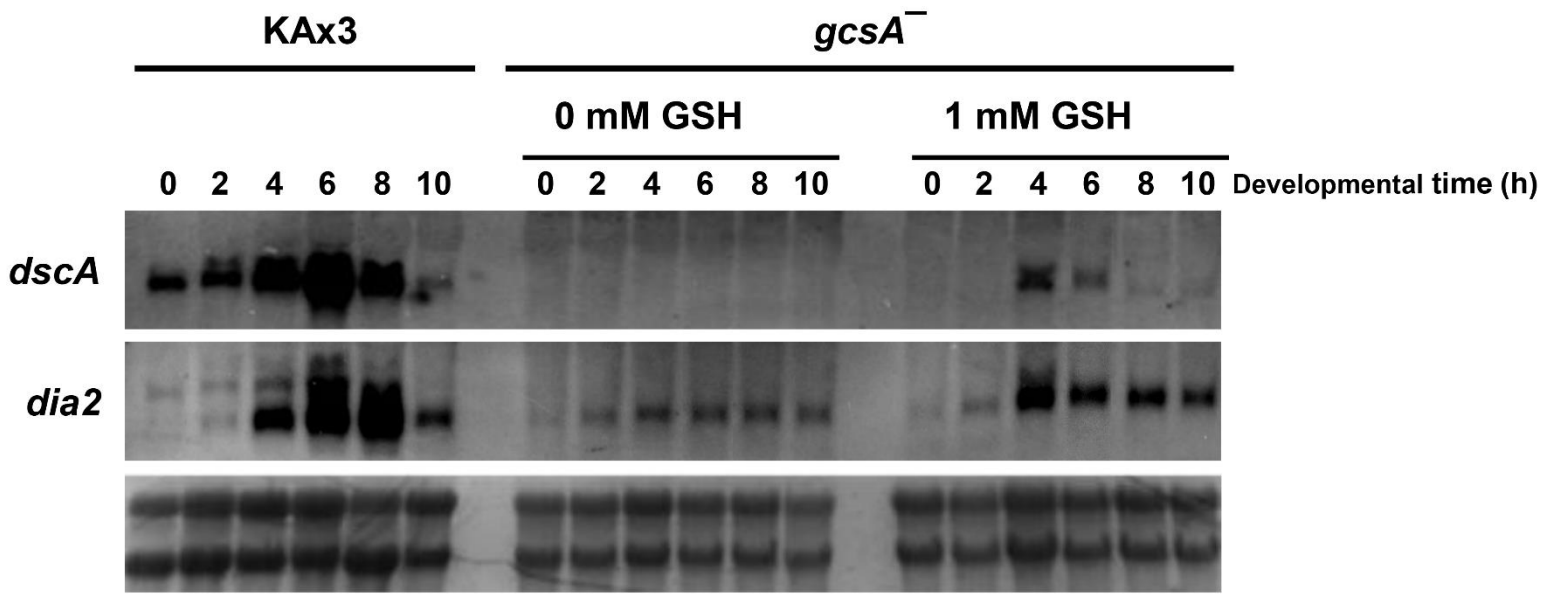


Fig. 10. Expression of *dscA* and *dia2* in *gcsA*$^{-}$ cells during suspension development. KAx3 and in *gcsA*$^{-}$ cells were allowed to develop in non-nutrient KK2 buffer. Developmental stage of in *gcsA*$^{-}$ cells in suspension was confirmed by analyzing the expression of *dscA* and *dia2*, which serves as markers for the initiation of development. *dscA*, encoding discoidin A; *dia2*, encoding novel lysine- and leucine-rich protein (DIA2).

signaling. The regulation of the expression of *carA* and *acaA* is required to initiate development (Anjard et al., 1992; Klein et al., 1988; Mann et al., 1992; Pitt et al., 1992). To investigate the cause of the developmental defects of GSH-depleted *gcsA*$^{-}$ cells, the expression of *carA* and *acaA* was examined by Northern blot analysis (Fig. 9). KAx3 and *gcsA*$^{-}$ cells were allowed to develop in suspension and total RNA samples were prepared at every 2 h intervals. The results showed that the expression of *carA* and *acaA* was induced in KAx3 cells. In contrast, the expression levels of *carA* and *acaA* were undetectably low in *gcsA*$^{-}$ cells in the absence of added GSH during the entire time suspension development. When 1 mM GSH was added to *gcsA*$^{-}$ cells, the expression pattern of *carA* and *acaA* was similar to that of KAx3 cells, although it was slightly decreased and delayed. These results indicate that GSH induces development through activating the expression of early developmental genes, particularly those involved in cAMP signaling in *Dictyostelium*.

3.2. The effect of cAMP stimulation on development of *gcsA*$^{-}$ cells

GSH-depleted *gcsA*$^{-}$ cells showed lack of gene expression related with the cAMP signaling cascade. Expression of ACA is one of the earliest responses of cells to starvation. This suggests that the absence cAMP signaling causes the aggregate-less phenotype of GSH-depleted *gcsA*$^{-}$ cells. The deficiency of cAMP oscillations because of the absence of ACA might be the defect in *gcsA*$^{-}$ cells. Insall *et al.* (1994) reported that that some aggregation-deficient mutants form aggregates and induce the expression of cAMP response genes when they are periodically stimulated with exogenous

cAMP. Thus, it was examined whether exogenously added cAMP pulses rescued the developmental defects of *gcsA*$^{-}$ cells. KAx3 and *gcsA*$^{-}$ cells were allowed to develop in suspension with nanomolar concentration of cAMP as described in materials and methods. According to the results, cAMP stimulation activates and accelerates formation of aggregates in both KAx3 and *gcsA*$^{-}$ cells with 1 mM GSH (Fig. 11). The aggregates were bigger and tighter than cAMP untreated cells, which are shown in Fig. 6. However, *gcsA*$^{-}$ cells were remained as single cells without the addition of GSH in spite of exogenously added cAMP pulses. For detailed analysis of developmental morphology, the progress of aggregation was observed by developmental time. *gcsA*$^{-}$ cells showed slightly late progression rate of aggregates formation by a few hours in comparison with KAx3 cells when they were subjected to non-nutrient buffer with 1 mM GSH (Fig. 12). Without GSH, *gcsA*$^{-}$ cells were in single cell state for 14 h in spite of the supplementation of cAMP pulses. In addition, the expression of *carA* and *acaA* was induced by exogenous cAMP stimulation in GSH-depleted *gcsA*$^{-}$ cells (data not shown). These results suggest that lack of cAMP secretion is not a main cause of aggregate-less phenotype of the GSH-depleted *gcsA*$^{-}$ cells.

3.3. The effect of cAR1 expression on development of *gcsA*$^{-}$ cells

Developmental defects of *gcsA*$^{-}$ cells were not explained by the absence of cAMP synthesis and secretion, because *gcsA*$^{-}$ cells did not develop and failed to induce the expression of *carA* and *acaA* without GSH even though exogenous cAMP was added periodically (Figs. 11 and 12).

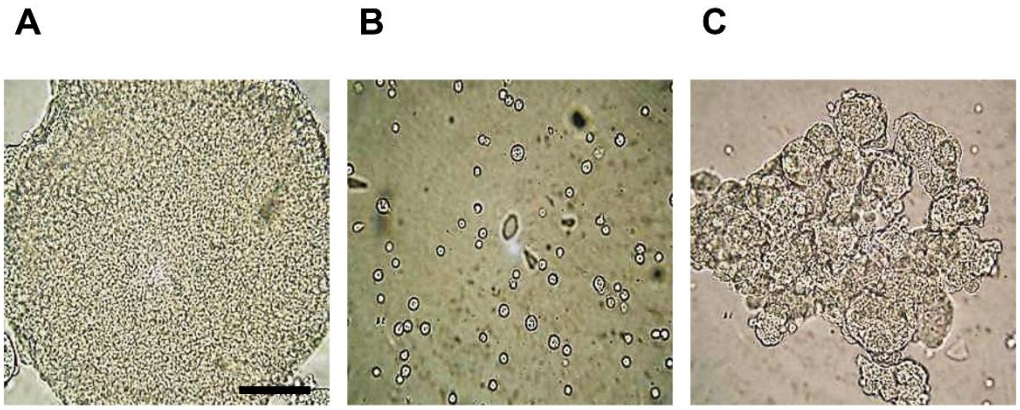


Fig. 11. Developmental morphology of KAx3 and *gcsA*$^{-}$ cells in suspension with cAMP pulses. KAx3 and *gcsA*$^{-}$ cells were allowed to develop in non-nutrient KK2 buffer with pulsed addition of nanomolar levels of cAMP and photographed at 12 h. (A) KAx3 cells, (B) *gcsA*$^{-}$ cells with no GSH, (C) *gcsA*$^{-}$ cells with 1 mM GSH. The scale bar represents 0.05 mm.

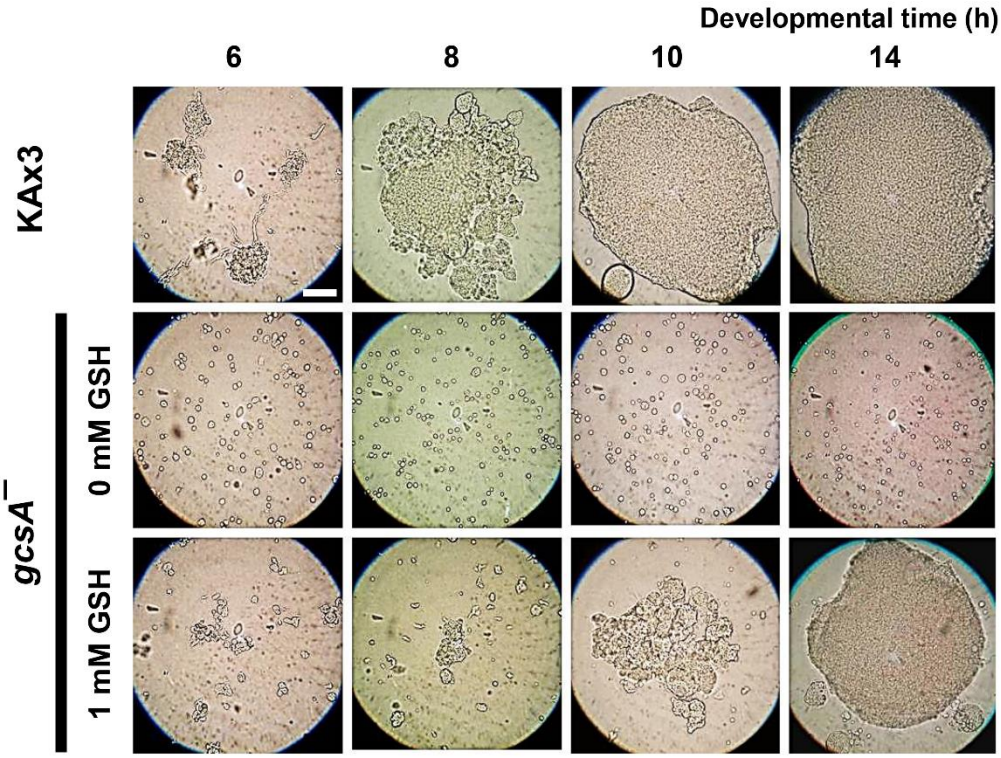


Fig. 12. Developmental morphology of KAx3 and *gcsA*¯ cells in suspension. KAx3 and *gcsA*¯ cells were allowed to develop in non-nutrient KK2 buffer with nanomolar levels of cAMP pulses. The developmental morphology was observed and photographed in time at 2 h intervals. The scale bar represents 0.05 mm.

Next, other possibility was considered that the failure of *gcsA*$^{-}$ cells to develop is reasoned by the defect in cAMP recognition. The cAMP receptor is required to induce development of *Dictyostelium*. Binding of cAMP to cAR1 is required for the activation of several second-messenger pathways, including G-protein-independent stimulation of calcium uptake, and G-protein dependent stimulation of adenylyl and guanylyl cyclases (Kesbeke *et al.*, 1988; Kumagai *et al.*, 1989; Milne and Coukell, 1991; Milne and Devreotes, 1993; Pupillo *et al.*, 1992; Sun *et al.*, 1990). As shown in Northern blotting analysis, *carA* encoding cAMP receptor (cAR1) was not expressed in *gcsA*$^{-}$ cells without the addition of 1 mM GSH (Fig. 9). Moreover, exogenous cAMP pulses failed to induce the expression of *carA* and failed to rescue the developmental defect of *gcsA*$^{-}$ cells. Thus, it was suspected that the failure of development in *gcsA*$^{-}$ might be due to the lack of cAR1 or deficiency of cAR1 activation.

It was determined whether constitutive expression of cAR1 under control of an actin promoter in *gcsA*$^{-}$ cells reversed the defects caused by depletion of GSH. Full-length of cAR1 was cloned into an integrating expression vector Exp4(+) under the control of *Actin15* promoter and the *act15::carA* expression construct was introduced to KAx3 and *gcsA*$^{-}$ cells (Fig. 13A). And the induced expression of cAR1 in both KAx3 and *gcsA*$^{-}$ cells was confirmed by Northern blotting (Fig. 13B). Developmental phenotype of cAR1-expressing KAx3 (cAR1^{OE}/KAx3) and *gcsA*$^{-}$ cells (cAR1^{OE}/*gcsA*$^{-}$) was observed when they were allowed to develop in suspension with cAMP pulses (Fig. 14). Although cAR1 was expressed, cAR1^{OE}/*gcsA*$^{-}$ cells did not develop in the absence of added GSH. The failure of *gcsA*$^{-}$ cells to develop was not attributed to the inability of cAR1

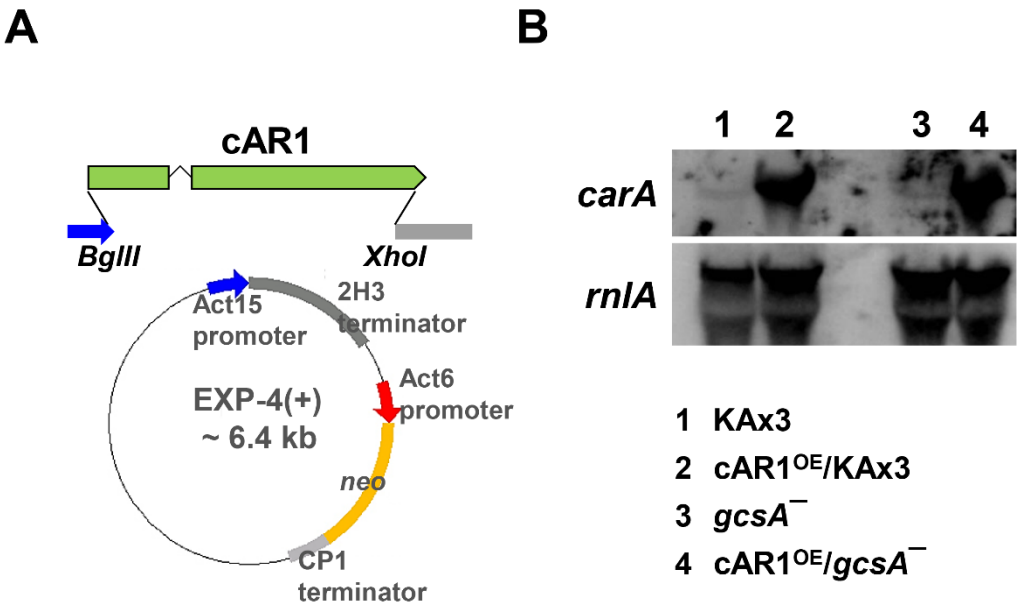


Fig. 13. Constitutive expression of cAR1 in KAx3 and *gcsA*$^{-}$ cells. (A) Preparation of the construct for cAR1 expression. Full-length of gDNA was cloned into integrating expression Exp4(+) vector containing constitutive *Act15* promoter. (B) Confirmation of cAR1 expression in KAx3 and *gcsA*$^{-}$ cells. The cAR1 expression in KAx3 and *gcsA*$^{-}$ cells was confirmed by analyzing the expression levels of *carA* mRNA by Northern blotting. *carA*, encoding cAMP receptor; *rnlA*, a loading control.

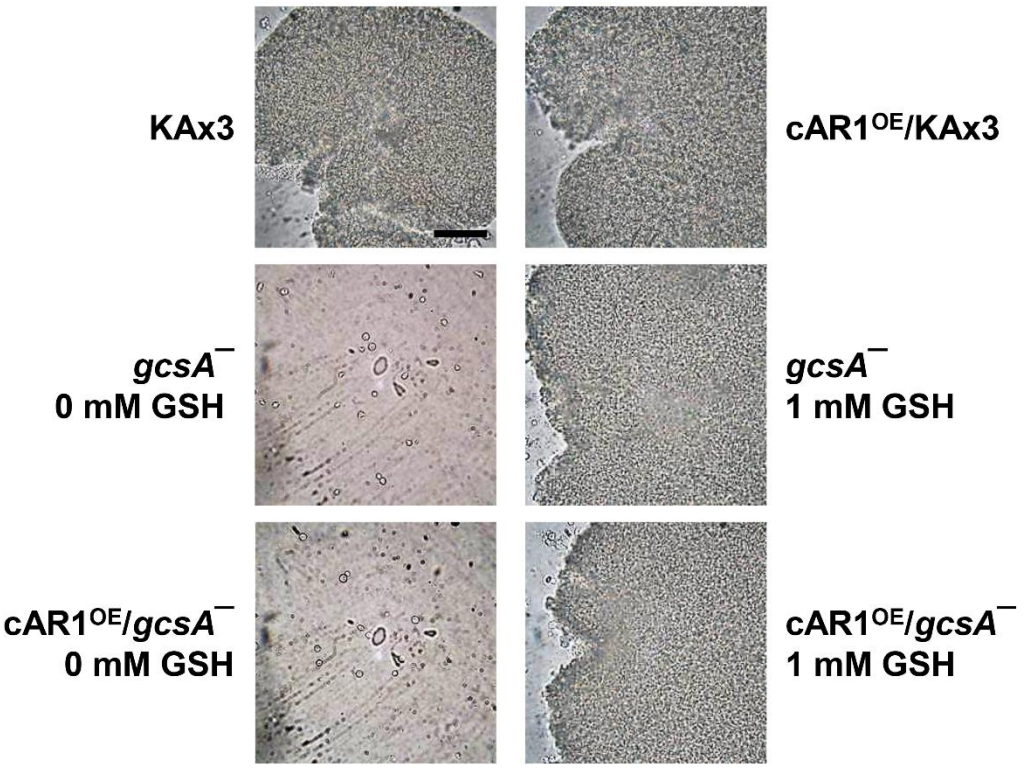


Fig. 14. Effect of cAR1 expression on the developmental morphology of *gcsA*$^{-}$ cells in suspension. cAR1-expressing KAx3 and *gcsA*$^{-}$ cells (cAR1^{OE}/KAx3 and cAR1^{OE}/*gcsA*$^{-}$ cells, respectively) were allowed to develop in non-nutrient KK2 buffer with cAMP pulses and photographed at 12h. The scale bar represents 0.05mm.

to respond to cAMP.

To determine whether the developmental defect in cAR1^{OE}/*gcsA*$^{-}$ cells was due to a deficiency in other components of the cAMP signaling pathway, the expression levels of *carA*, *acaA*, *gpaB* (G-protein alpha subunit 2), *pkaC*, and *pkaR* were determined. It was found that the expression of *acaA* and *gpaB* was induced by the constitutive expression of *carA* in *gcsA*$^{-}$ cells (cAR1^{OE}/*gcsA*$^{-}$) without GSH (Fig. 15). However, the transcriptional expression of *pkaC* and *pkaR* was not affected significantly by the constitutive expression of cAR1. In other words, *gcsA*$^{-}$ cells did not form aggregates in the absence of GSH when cAR1 was expressed, although cAMP signaling was activated at a functional level. These results suggest that GSH interacts with other pathway which functions at earlier step than cAMP signaling to regulate the transition from growth to development.

4. The role of GSH in the regulation of YakA signaling

4.1. The expression of *yakA* in *gcsA*$^{-}$ cells

gcsA^{-} cells showed aggregate-less phenotype and abnormal transcriptional regulation of early developmental genes, especially *carA* and *acaA* which are important components of the cAMP signaling system. And the restoration of *carA* and *acaA* expression by cAMP stimulation and cAR1 expression did not rescue the developmental defect of *gcsA*$^{-}$ cells. Activation of the YakA signaling pathway is known the earliest developmental regulatory event before cAMP signaling occurs. When cells are starved, YakA inhibits the expression of vegetative-state-specific gene, in particular

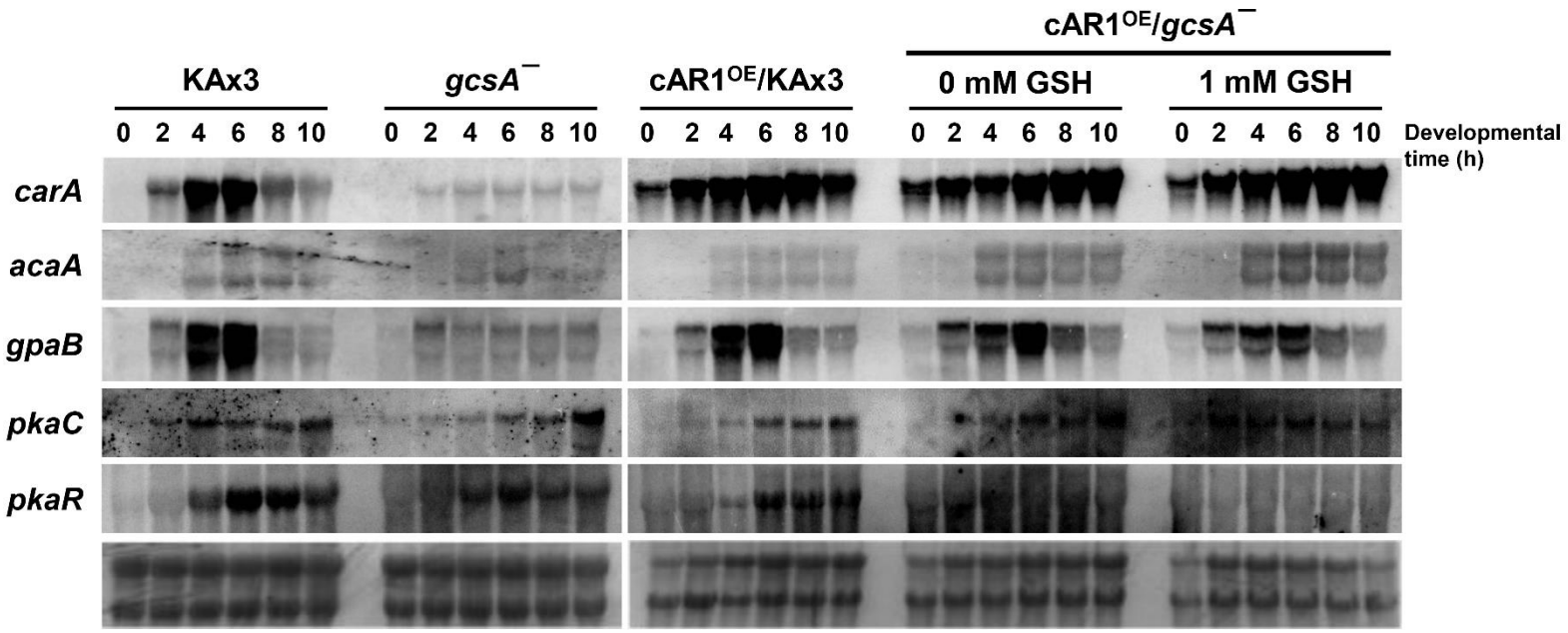


Fig. 15. Effect of cAR1 expression on developmental gene expression. KAx3, *gcsA*$^{-}$, cAR1^{OE}/KAx3, and cAR1^{OE}/*gcsA*$^{-}$ cells were allowed to develop in non-nutrient KK2 buffer with cAMP pulses. The expression of genes related to the cAMP signaling pathway was analyzed by Northern blotting. *carA*, encoding cAMP receptor 1; *acaA*, encoding adenylyl cyclase A; *gpaB*, encoding G-protein alpha subunit 2; *pkaC*, encoding cAMP-dependent protein kinase A catalytic subunit; *pkaR*, encoding cAMP-dependent protein kinase A regulatory subunit.

to reduce *pufA* expression, which inhibits translation of *pkaC* mRNA (Souza et al., 1999) and induces an increase in the expression of aggregation-state-specific genes, such as *carA* and *acaA* (Souza et al., 1998).

To investigate the relationship between GSH and the YakA, the expression of *yakA* was determined in *gcsA*$^{-}$ cells. The transcription of *yakA* was induced as the nutrient exhausted and reach a maximum at 6 h, and after then fell slowly in KAx3 cells (Fig. 16). The level of *yakA* expression in the *gcsA*$^{-}$ cells remained very low during development in suspension without the addition of GSH, which was similar levels to that of *yakA*$^{-}$ cells. Interestingly, the expression of *yakA* was induced by adding 1 mM GSH to *gcsA*$^{-}$ cells; however, the levels were slightly lower than those in KAx3 cells. These findings indicate that the expression of *yakA* may be regulated by the intracellular GSH.

4.2. The effect of intracellular GSH on the expression of *yakA*

According to above results, the expression of *yakA* seems to be regulated by intracellular GSH to initiate development of *Dictyostelium*. These results were confirmed by analyzing the expression of *yakA* in KAx3 cells when exposed to diverse concentration of GSH. KAx3 cells were allowed to develop with 0 mM, 0.5 mM, 1.0 mM, 2.0 mM and 3.0 mM of GSH in non-nutrient KK2 buffer. As the concentration of GSH increased, the expression of *yakA* increased proportionally and reached a peak more rapidly compared to that with the control (Fig. 17). Aggregation started slightly faster in proportion to the concentration of GSH (Fig. 18). Exogenously added GSH induced the expression of *yakA*, and to demonstrate these results

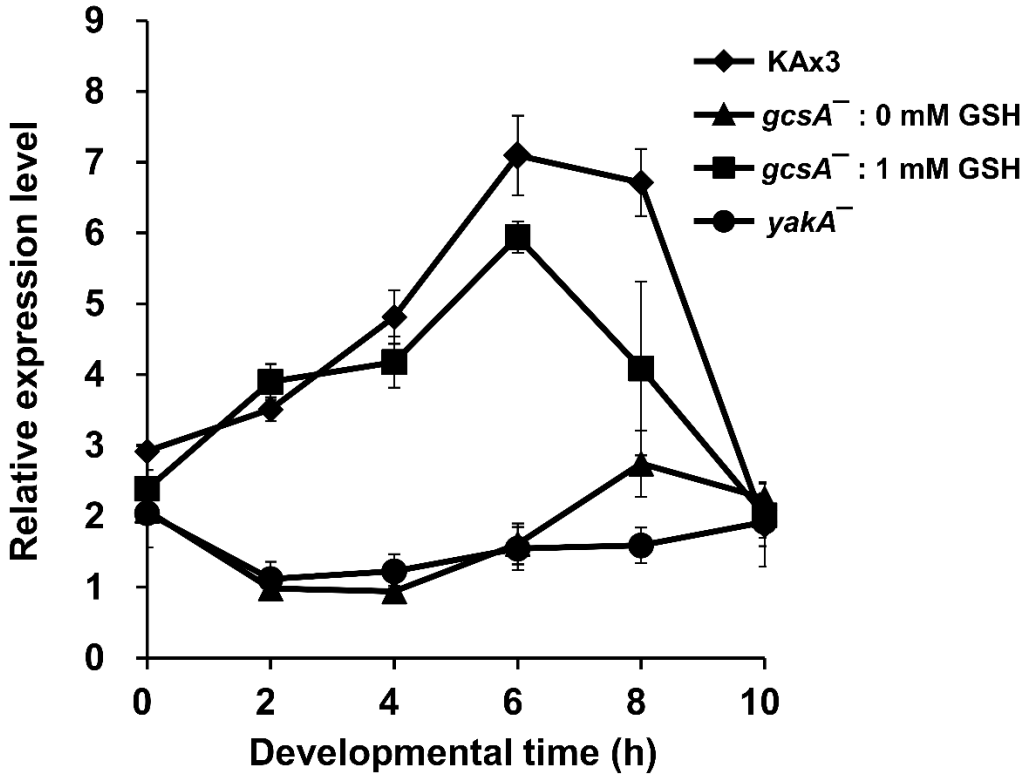


Fig. 16. Expression levels of *yakA* in KAx3 and *gcsA*$^{-}$ cells during development in suspension. KAx3, *gcsA*$^{-}$, and *yakA*$^{-}$ cells were allowed to develop in non-nutrient KK2 buffer for 10 h, and total RNA was extracted at 2 h intervals. The expression of *yakA* was analyzed using real-time RT-PCR. All expression data were normalized by dividing the amount of *yakA* by the amount of *rnlA* used as a control. The values represent mean ± S.E.M. of three independent experiments. *yakA*, protein serine/threonine kinase.

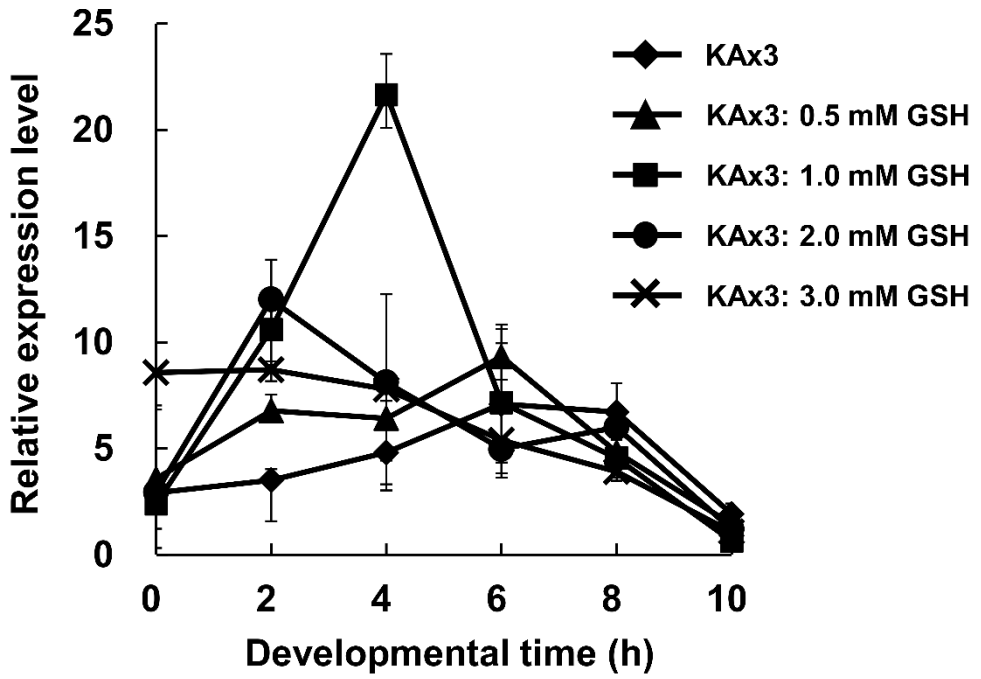


Fig. 17. Effect of exogenous GSH on the expression patterns of *yakA* during development in suspension. KAx3 cells were allowed to develop in non-nutrient KK2 buffer for 10 h with diverse concentration of GSH; 0 mM, 0.5 mM, 1.0 mM, 2.0 mM, and 3.0 mM of GSH. Total RNA was extracted at 2 h intervals. The expression of *yakA* was analyzed using real-time RT-PCR. All expression data were normalized by dividing the amount of *yakA* by the amount of *rnlA* used as a control. The values represent mean ± S.E.M. of three independent experiments. *yakA*, protein serine/threonine kinase.

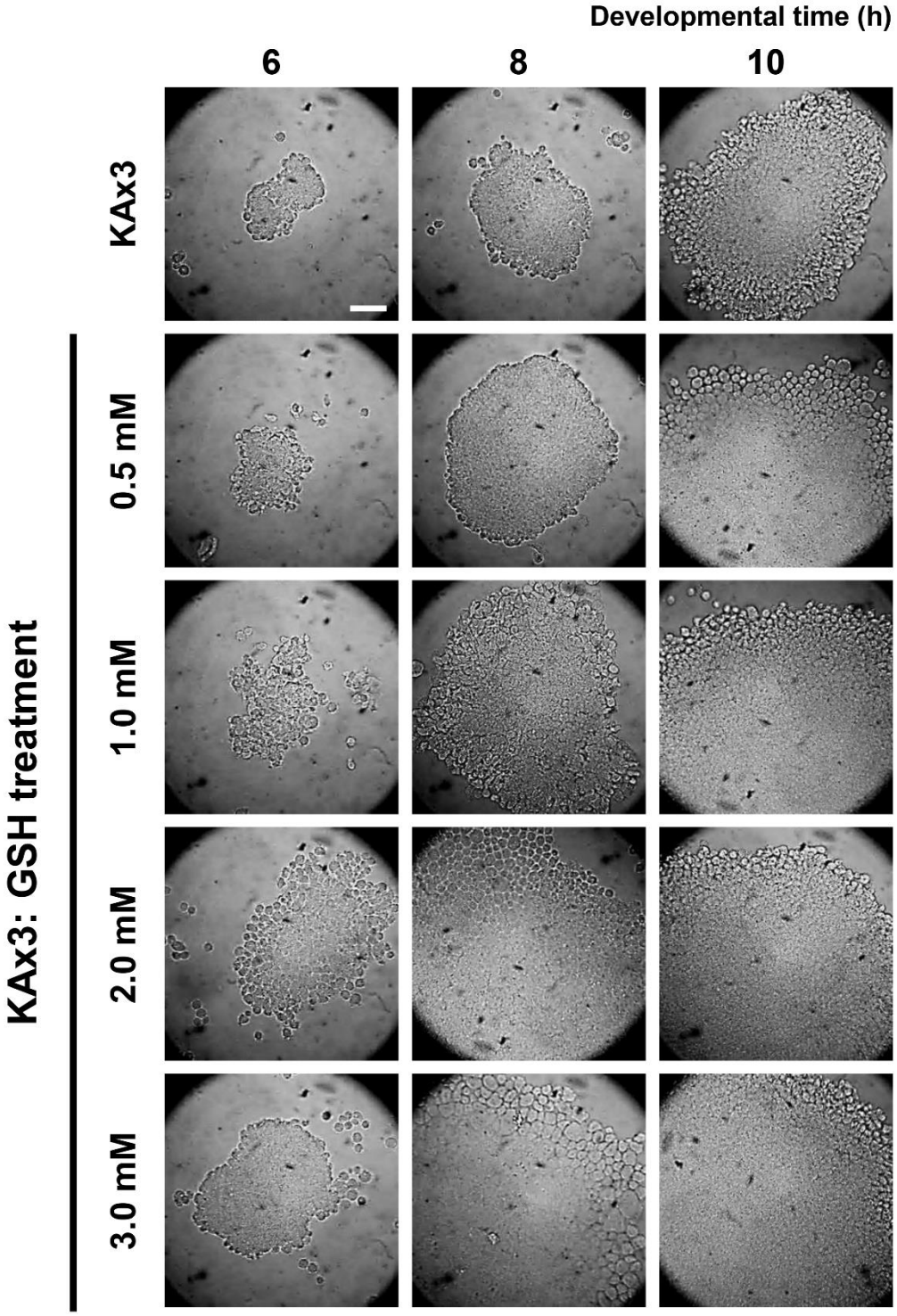

Developmental time (h)
6
8
10
KAx3
KAx3: GSH treatment
0.5 mM
1.0 mM
2.0 mM
3.0 mM

Fig. 18. Effect of exogenous GSH on the progress of the formation of aggregates. KAx3 cells were allowed to develop in non-nutrient KK2 buffer for 10 h in the presence of 0 mM, 0.5 mM, 1.0 mM, 2.0 mM, and 3.0 mM GSH and photographed at the indicated time. The scale bar represents 0.05 mm.

further, the expression of *yakA* and developmental morphology were observed in GCS-overexpressing KAx3 cells (GCS^{OE}/KAx3). GCS^{OE}/KAx3 cells showed significantly increased intrinsic GSH contents more than 160% compared to that of KAx3 cells (Fig. 19). The same events as the *yakA* expression and developmental morphology in KAx3 cells treated with exogenous GSH occurred in GCS^{OE}/KAx3. The expression of *yakA* was considerably increased and reached the peak 2 h earlier than that of KAx3 cells (Fig. 20). The formation of aggregates also occurred at a slightly faster rate than in KAx3 cells (Fig. 21). These results indicate that GSH regulates the transition from growth to development by regulating the expression of *yakA*.

4.3. The expression of YakA downstream regulators in *gcsA*$^{-}$ cells

yakA was not expressed appropriately without GSH. It was assumed that diminished expression of *yakA* caused blocked developmental life cycle in *gcsA*$^{-}$ cells. YakA signaling system is composed of YakA, PufA, PKA, and ACA. The expression of downstream regulators of YakA signaling cascade was also monitored.

4.3.1. The expression of *pufA*

First, the expression of *pufA* was analyzed by Northern blotting. PufA is a translational inhibitor of PKA-C and transcription of *pufA* is regulated by YakA (Souza *et al.*, 1999). Increased YakA during development inhibits its transcription and eventually resulted in the increased PKA activity. The results showed that the expression of *pufA* increased in GSH-depleted *gcsA*$^{-}$

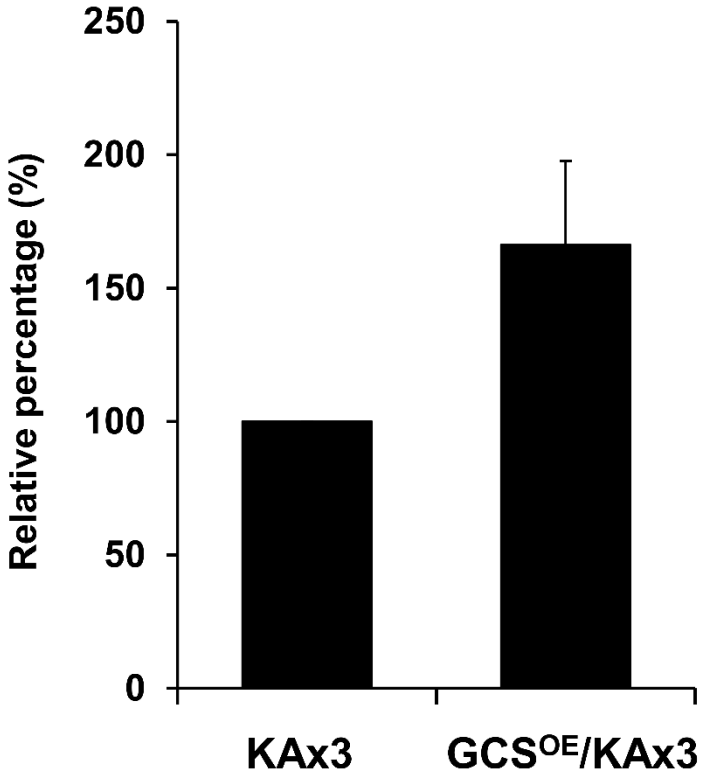


Fig. 19. Intracellular GSH concentration of GCS^{OE}/KAx3 cells. The concentration of intracellular GSH was measured in exponentially growing KAx3 and GCS^{OE}/KAx3 cells using HPLC and fluorescent detector. Intracellular GSH was modified to a mBBr-conjugated form to detect. The concentration of GSH was calculated in relative values compared to that of KAx3 cells. The values represent the mean ± S.E.M. of three independent experiments.

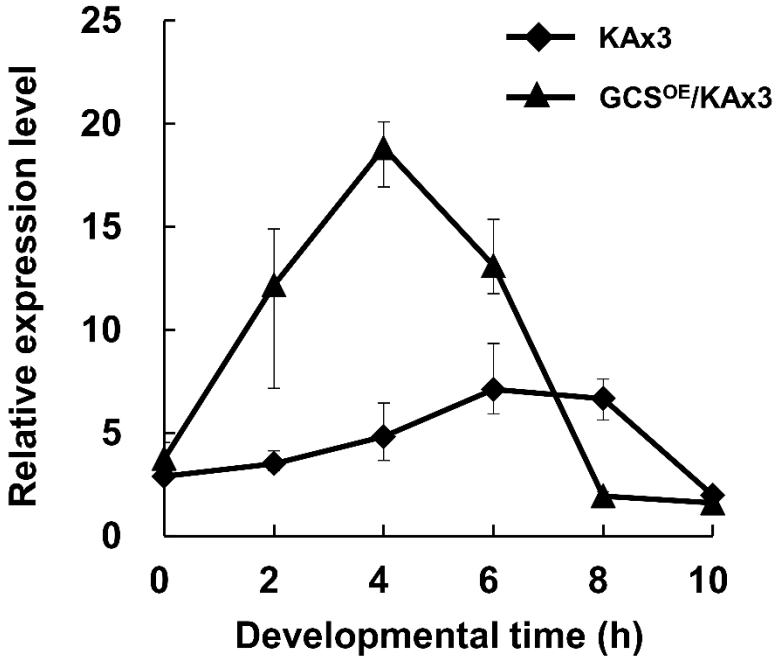


Fig. 20. Effect of the constitutive expression of GCS in KAx3 cells on the expression patterns of *yakA* during development in suspension. KAx3 and GCS^{OE}/KAx3 cells were allowed to develop in non-nutrient KK2 buffer for 10 h and total RNA was extracted at 2 h intervals. The expression of *yakA* was analyzed using real-time RT-PCR. All expression data were normalized by dividing the amount of *yakA* by the amount of *rnlA* used as a control. The values represent the mean ± S.E.M. of three independent experiments. *yakA*, protein serine/threonine kinase.

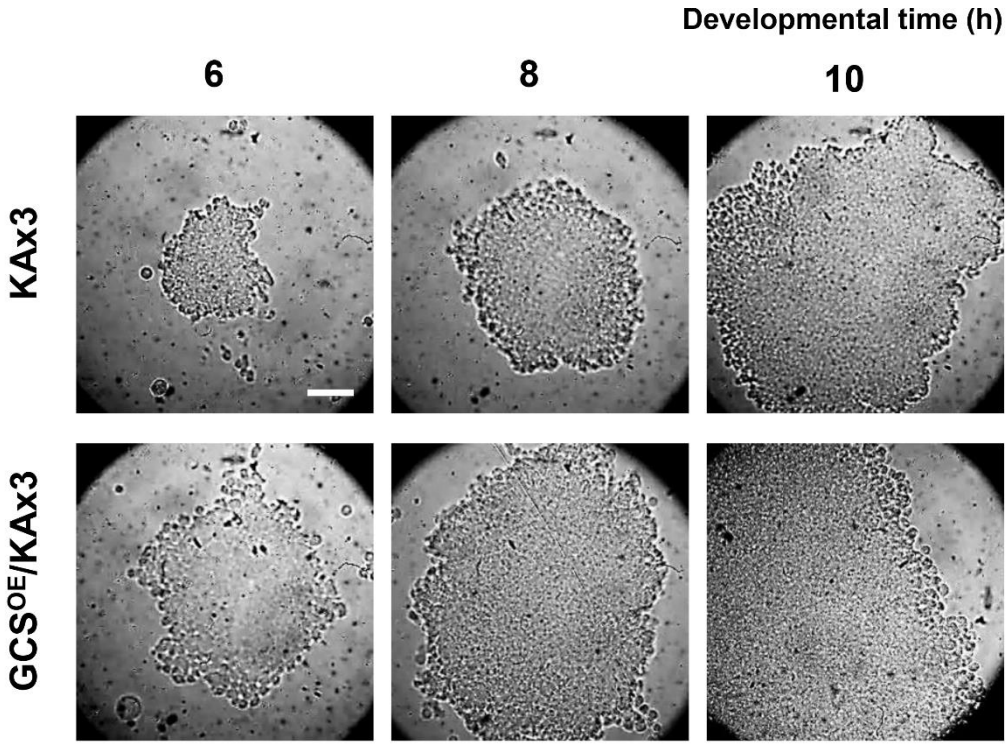


Fig. 21. Developmental morphology of GCS^{OE}/KAx3 cells in suspension. KAx3 and GCS^{OE}/KAx3 cells were allowed to develop in non-nutrient KK2 buffer with cAMP pulses for 10 h and photographed at the indicated time. The scale bar represents 0.05 mm.

cells and the supplementation of GSH repressed the expression of *pufA* in *gcsA*$^{-}$ cells (Fig. 22). These results are consistent with the lowered *yakA* expression in *gcsA*$^{-}$ cells.

4.3.2. The gene expression and the enzymatic activity of PKA in *gcsA*$^{-}$ cells

Next, transcriptional expression of *pkaC* and the enzymatic activity of PKA were determined in *gcsA*$^{-}$ cells because the expression of *pufA*, which inhibits PKA-C translation, increased in *gcsA*$^{-}$ cells (Fig. 22). According to the Northern blotting results, the expression of *pkaC*, a catalytic subunit of PKA, was not extensively affected by intracellular GSH (Fig. 22). *pkaC* was expressed in *gcsA*$^{-}$ cells without GSH with similar level to KAx3 cells and *gcsA*$^{-}$ cells with 1 mM GSH. However, PKA activity was much lower in *gcsA*$^{-}$ cells without the addition of GSH than KAx3 cells (Fig. 23). In contrast, the activity of PKA recovered to similar levels compared with KAx3 cells when 1 mM GSH was added. The lowered activity of PKA in GSH-depleted *gcsA*$^{-}$ cells was consistent with the decreased *yakA* expression and the increased *pufA* expression shown in Figs. 16 and 21, respectively. These results indicate that intracellular GSH is needed to activate the YakA signaling pathway which is required for the transition from growth to development.

5. Developmental properties of *yakA*$^{-}$ cells

5.1. The developmental morphology of *yakA*$^{-}$ cells

To explain the relation between YakA and GSH more, developmental

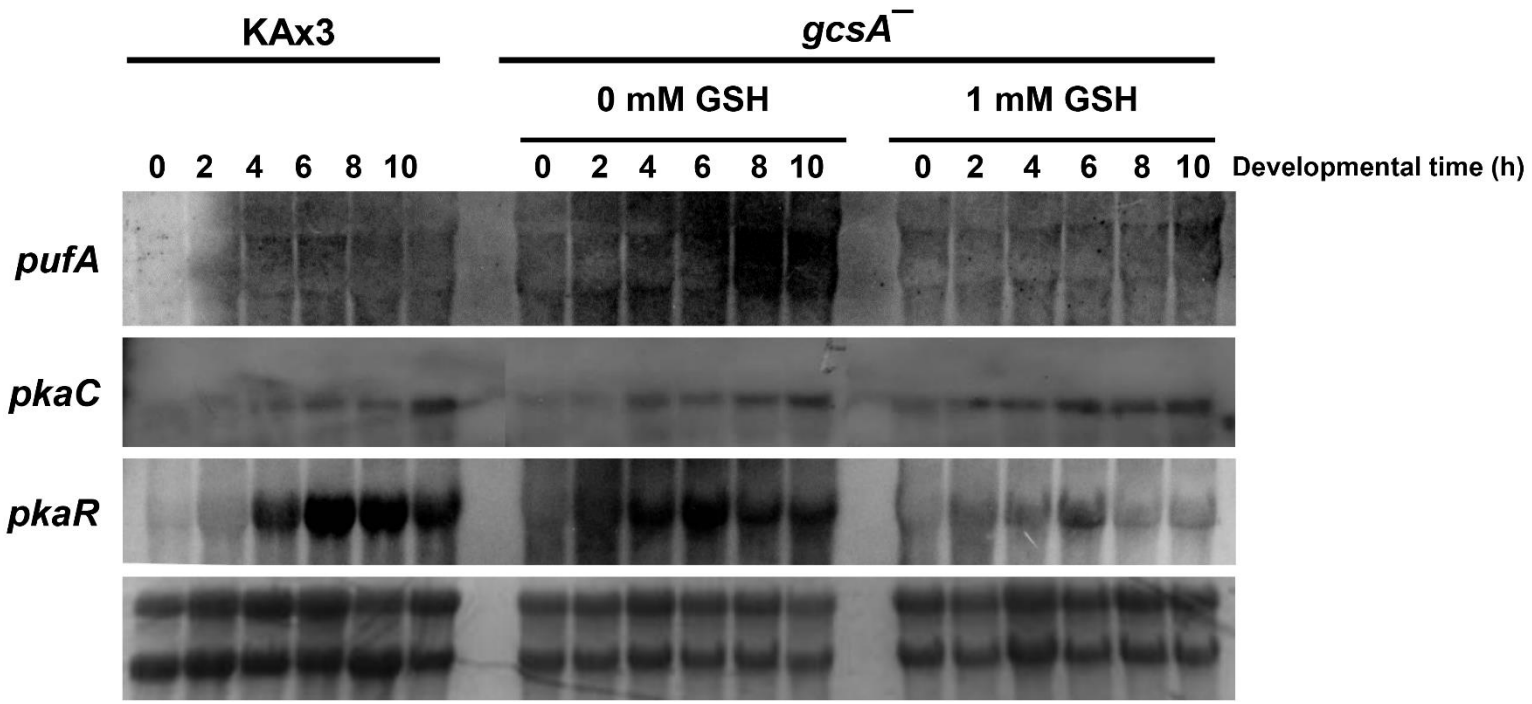


Fig. 22. Expression of downstream regulators of the YakA signaling system in KAx3 and *gcsA*¯ cells. KAx3 and *gcsA*¯ cells were allowed to develop in non-nutrient KK2 buffer with cAMP pulses. The expression of genes encoding downstream regulators of the YakA signaling pathway was analyzed by Northern blotting. *pufA*, encoding a RNA binding protein; *pkaC*, encoding protein kinase catalytic subunit; *pkaR*, encoding protein kinase regulatory subunit.

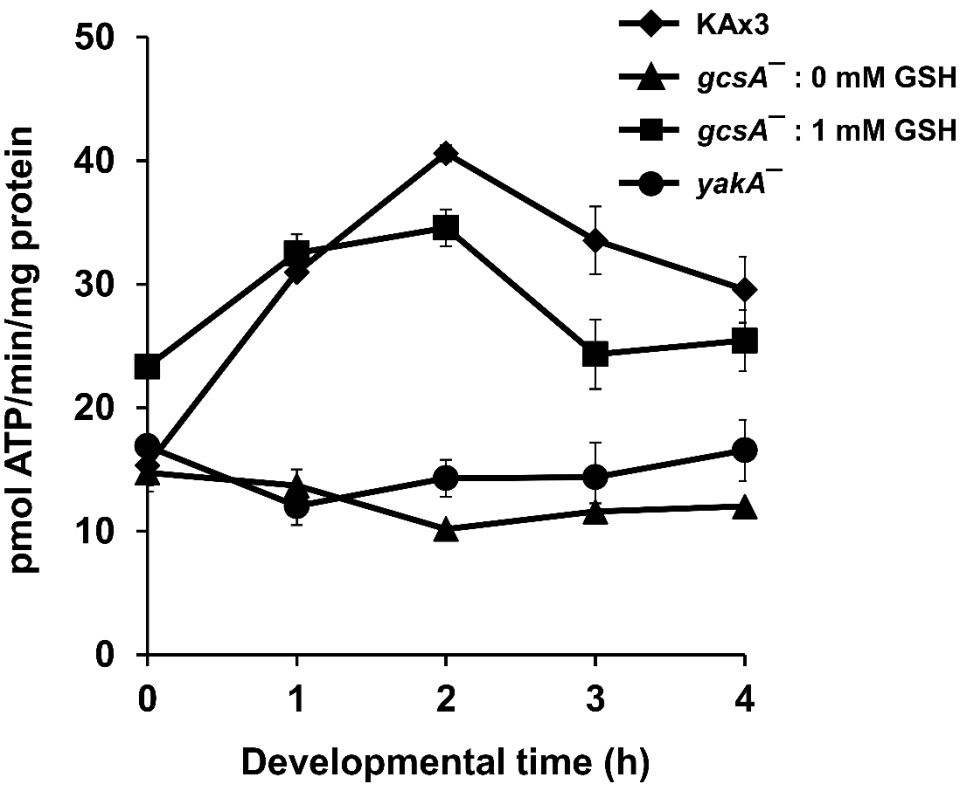


Fig. 23. PKA activity in KAx3, *gcsA*¯, and *yakA*¯ cells during development in suspension. KAx3, *gcsA*¯, and *yakA*¯ cells were allowed to develop in non-nutrient KK2 buffer with cAMP pulses. The activity of PKA was measured using the SignaTECT PKA Activity System (Promega). The values represent the mean ± S.E.M. of three independent experiments.

morphology of *yakA*$^{-}$ cells was observed. As previously reported by Souza *et al.* (1998), *yakA*$^{-}$ cells were completely deficient in the formation of aggregation when they were placed on non-nutrient agar plates (data not shown). To analyze the developmental state of *yakA*$^{-}$ cells, *yakA*$^{-}$ cells were induced to develop in suspension. When suspended in non-nutrient KK2 buffer, they existed as single cells as *gcsA*$^{-}$ cells (Fig. 24). These results suggest that *gcsA*$^{-}$ cells and *yakA*$^{-}$ could not develop because they do not express *yakA* in response to developmental environment.

5.2. The expression of developmental genes in *yakA*$^{-}$ cells

Interestingly, *yakA*$^{-}$ cells showed remarkably similar patterns of early developmental gene expression and PKA activity compared with *gcsA*$^{-}$ cells. The expression of *carA*, *acaA*, and *pkaR* decreased significantly. In contrast, the expression of *pufA* increased significantly in GSH-depleted *gcsA*$^{-}$ and *yakA*$^{-}$ cells compared with KAx3 cells (Fig. 25). The activity of PKA was also decreased in both *gcsA*$^{-}$ and *yakA*$^{-}$ cells (Fig. 23). These results show that low expression levels of *yakA* and its downstream regulators and developmental defects are found in both *gcsA*$^{-}$ and *yakA*$^{-}$. Taken together, these results suggest that the developmental defects of *gcsA*$^{-}$ cells are caused by the lack of *yakA* expression which is essential for the initiation of development by activating PKA and triggers the expression of *carA* and *acaA*.

5.3. The effect of GSH on the developmental morphology of *yakA*$^{-}$ cells

It was examined that whether the supplementation of GSH rescued the

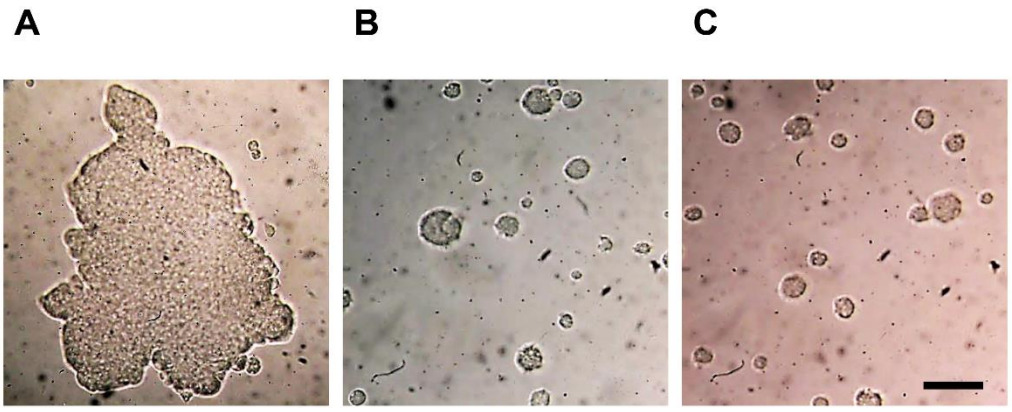


Fig. 24. Developmental morphology of *yakA*$^{-}$ cells in suspension. KAx3 and *yakA*$^{-}$ cells were allowed to develop in non-nutrient KK2 buffer with periodically added cAMP pulses and photographed at 12 h. (A) KAx3 cells, (B) *yakA*$^{-}$ cells, (C) *gcsA*$^{-}$ cells with no GSH. The scale bar represents 0.05 mm.

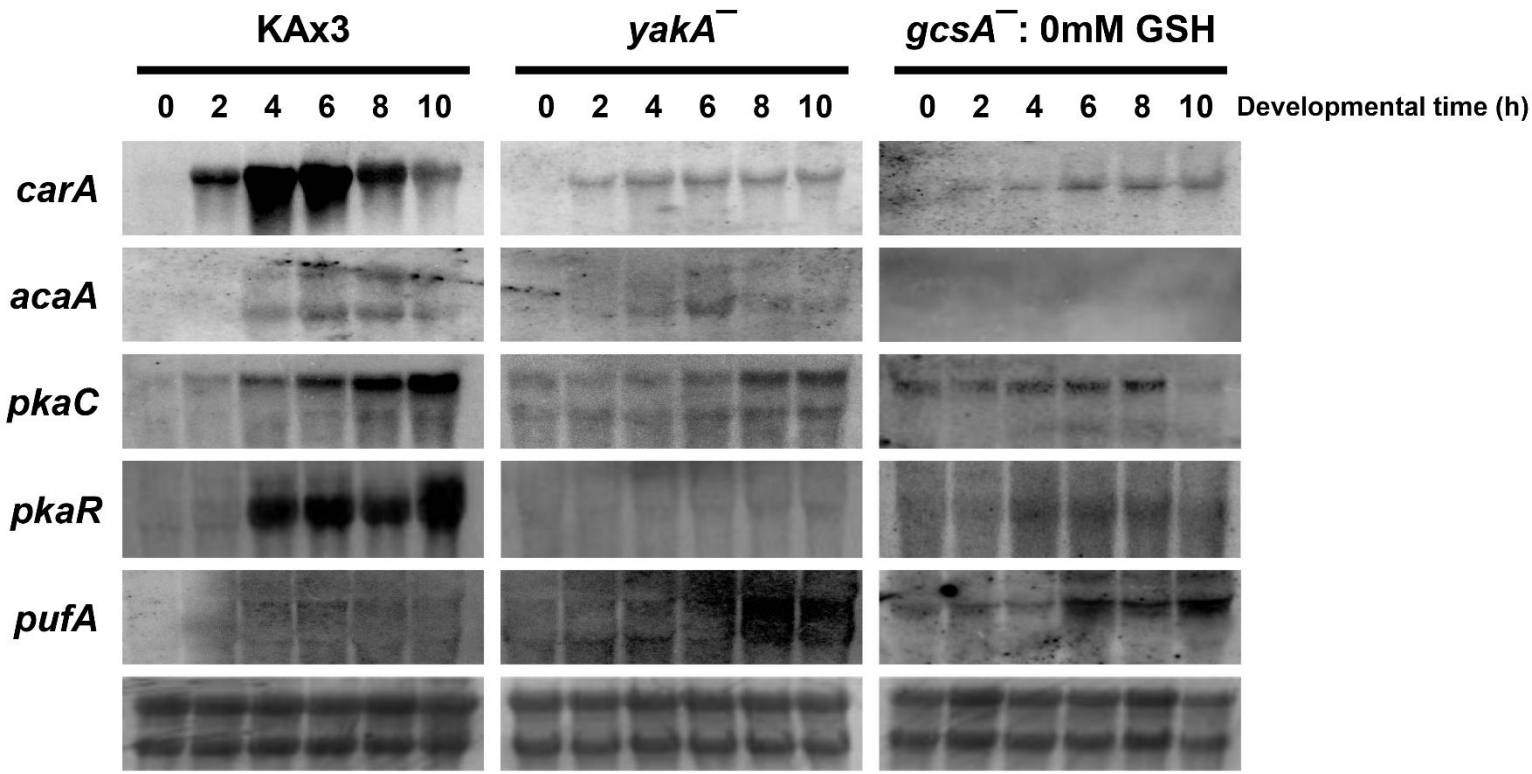

KAx3
yakA¯
gcsA¯: 0mM GSH
0 2 4 6 8 10
0 2 4 6 8 10
0 2 4 6 8 10
Developmental time (h)
carA
acaA
pkaC
pkaR
pufA

Fig. 25. Expression of developmental genes in *yakA*$^{-}$ cells. . KAx3, *gcsA*$^{-}$, and *yakA*$^{-}$ cells were allowed to develop in non-nutrient KK2 buffer. The expression patterns of several developmental genes were analyzed in *yakA*$^{-}$ cells by Northern blotting and compared with the expressions of KAx3 and *gcsA*$^{-}$ cells. *carA*, encoding cAMP receptor 1; *acaA*, encoding adenylyl cyclase A; *pkaC*, encoding protein kinase catalytic subunit; *pkaR*, encoding protein kinase regulatory subunit; *pufA*, encoding a RNA binding protein.

developmental defect of *yakA*$^{-}$ cells as did in *gcsA*$^{-}$ cells. *yakA*$^{-}$ cells were induced to develop with cAMP pulses and 1 mM GSH in non-nutrient KK2 buffer. They did not develop and existed as single cells though GSH was added (Fig. 26). These results imply that GSH regulates the initiation of development by activating the expression of *yakA* in *Dictyostelium*.

6. The role of GSH in the regulation of YakA signaling

6.1. The effect of YakA expression on the developmental morphology of *gcsA*$^{-}$ cells

The results so far achieved propose that GSH obviously has role in the life cycle shift from growth to development in *Dictyostelium* by regulating the expression of *yakA* and the aggregate-less phenotype of *gcsA*$^{-}$ cells is explained by the absence of *yakA* expression. To support these results, it was investigated whether constitutive expression of YakA could restore the defects in *gcsA*$^{-}$ cells. YakA was continuously expressed under the control of the actin15 promoter in KAx3 (YakA^{OE}/KAx3) and *gcsA*$^{-}$ (YakA^{OE}/*gcsA*$^{-}$) cells. When they were allowed to develop in suspension, YakA^{OE}/*gcsA*$^{-}$ cells formed aggregates which were similar to those of KAx3 cells regardless of the addition of GSH (Fig. 27). Interestingly, YakA^{OE}/KAx3 cells showed slightly faster rate of aggregation process compared to KAx3 cells (Fig. 28). YakA^{OE}/*gcsA*$^{-}$ cells developed in a comparable rate with KAx3 cells without GSH. These results confirm that GSH promotes induction of *yakA* expression to initiate development in *Dictyostelium*.

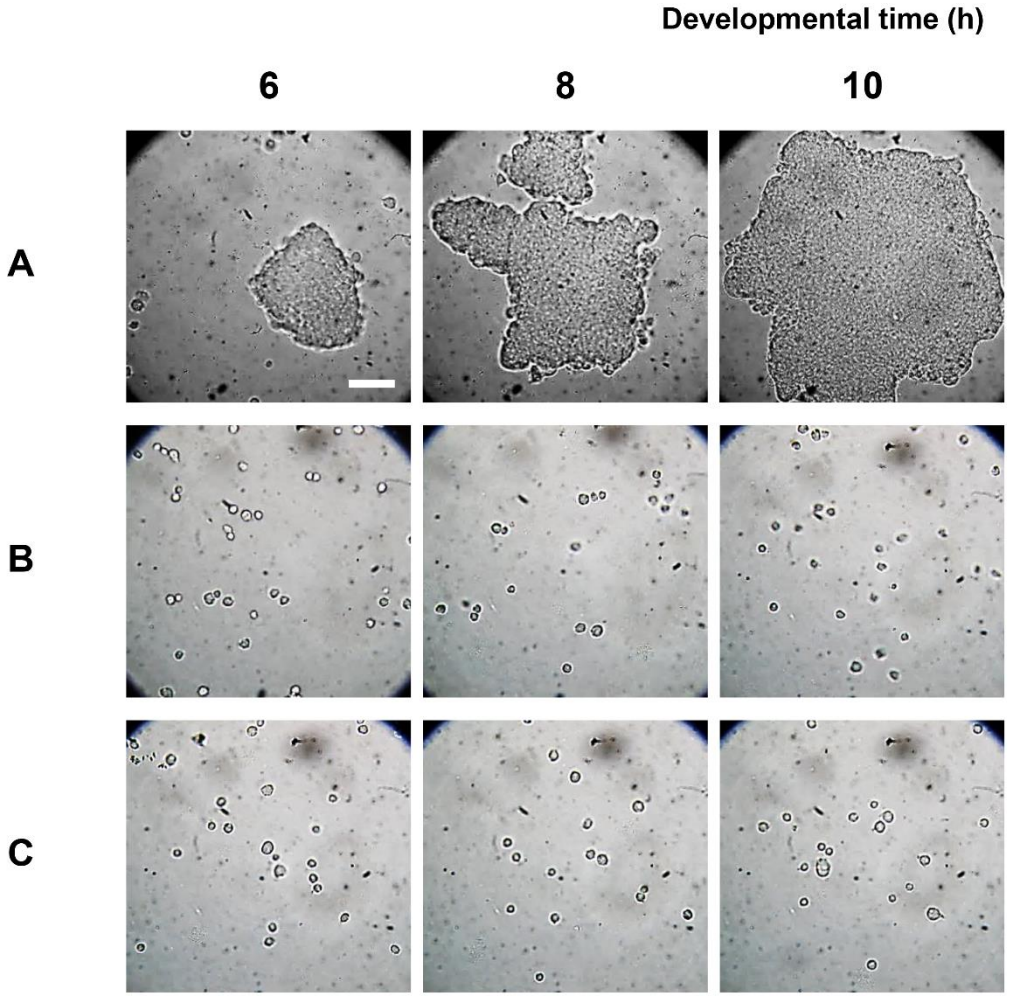


Fig. 26. Effect of GSH on development of *yakA*$^{-}$ cells in suspension. KAx3 and *yakA*$^{-}$ cells were subjected in non-nutrient KK2 buffer with nanomolar levels of cAMP pulses and 1 mM GSH. The progress of aggregation was observed for 10h and photographed at the indicated time. (A) KAx3 cells, (B) *yakA*$^{-}$ cells without GSH, (C) *yakA*$^{-}$ cells with 1 mM GSH. The scale bar represents 0.05 mm.

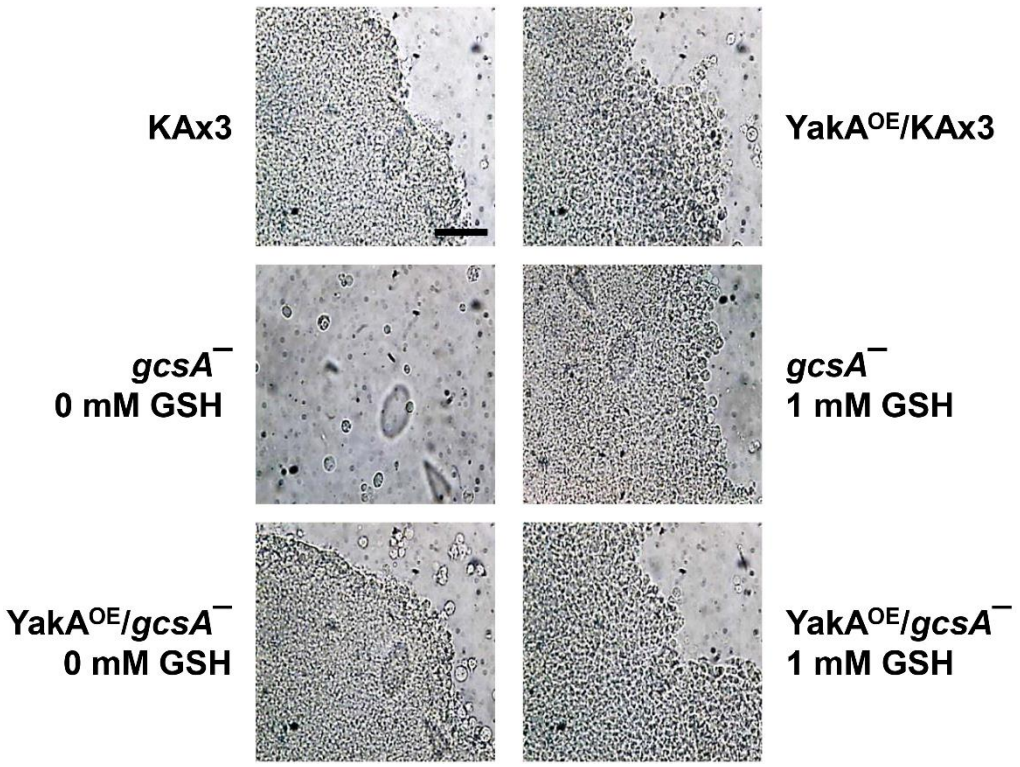


Fig. 27. Effect of YakA expression in *gcsA*$^{-}$ cells on developmental morphology. YakA-expressing KAx3 and *gcsA*$^{-}$ cells (YakA^{OE}/KAx3 and YakA^{OE}/*gcsA*$^{-}$ cells, respectively) were allowed to develop in non-nutrient KK2 buffer with exogenously added cAMP pulses and photographed at 12h. The scale bar represents 0.05mm.

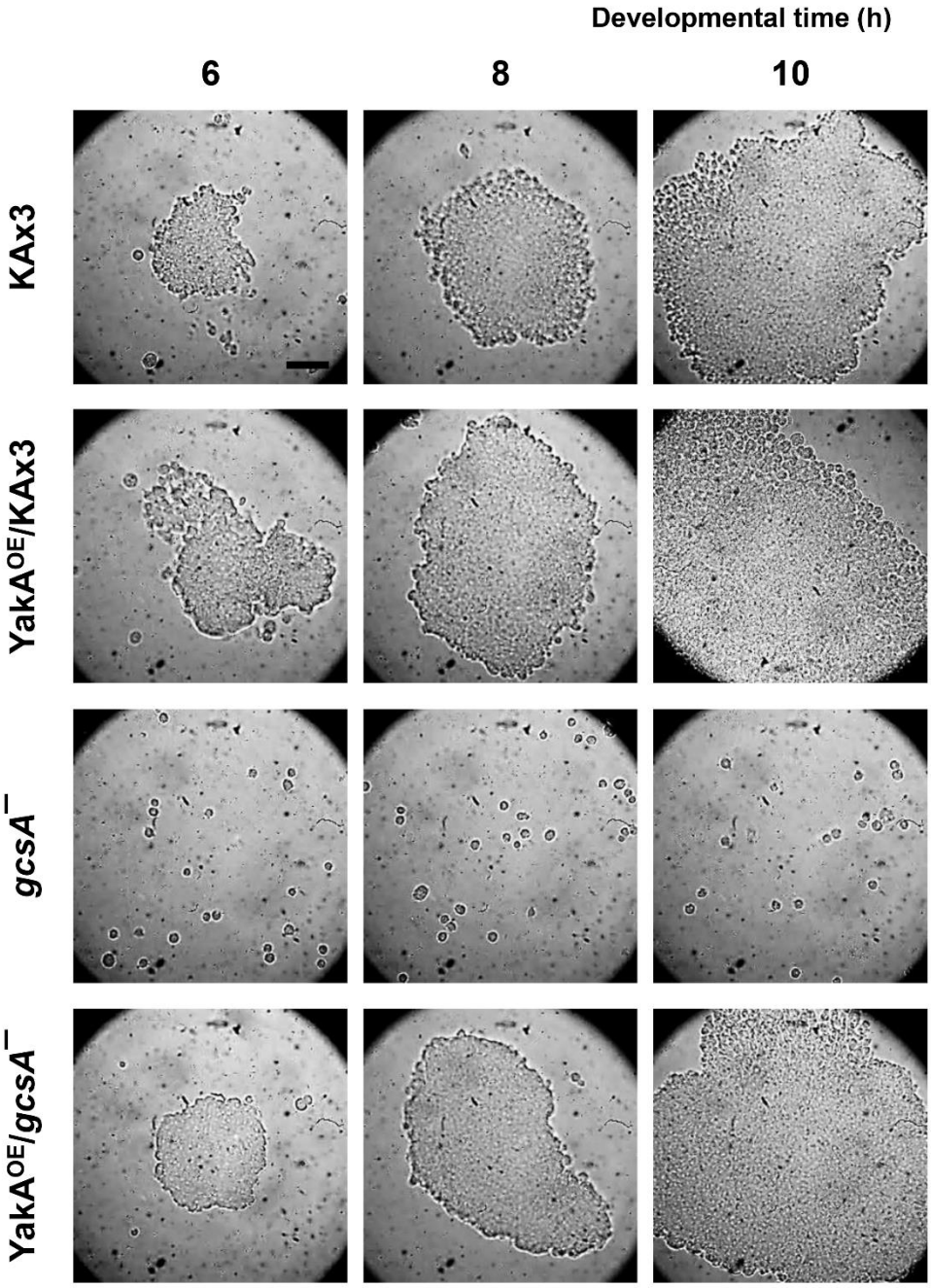


Fig. 28. Effect of YakA expression on the progress of aggregation. KAx3, YakA^{OE}/KAx3, *gcsA*$^{-}$, and YakA^{OE}/*gcsA*$^{-}$ cells were allowed to develop in non-nutrient KK2 buffer for 10 h with cAMP pulses and photographed at the indicated time. The scale bar represents 0.05 mm.

6.2. The effect of YakA expression on the expression of early developmental genes in *gcsA*$^{-}$ cells

gcsA^{-} cells developed without the addition of GSH by expressing YakA constitutively. Constitutive expression of YakA also influences the expression of early developmental genes in *gcsA*$^{-}$ cells. The expression of *carA*, *acaA*, and *pkaC* was significantly increased in *gcsA*$^{-}$ cells by the constitutive expression of YakA in *gcsA*$^{-}$ cells (YakA^{OE}/*gcsA*$^{-}$), although GSH was not added (Fig. 29). Further, the levels of *pufA* expression decreased in YakA^{OE}/*gcsA*$^{-}$ cells without the addition of GSH. Interestingly, the expression of *carA*, *acaA*, and *pkaC* also was significantly higher in YakA^{OE}/KAx3 cells than in *yakA*$^{-}$ cells (Fig. 29). These results demonstrate that the developmental defects in GSH-depleted *gcsA*$^{-}$ cells are due to the decreased expression of *yakA*. Thus, YakA expression rescues the expression of early developmental genes and eventually leads to proper development.

6.3. The effect of YakA expression on the concentration of intracellular GSH

To define the relation between YakA and GSH in *Dictyostelium*, intracellular concentration of GSH and total glutathione, which is sum of the GSH and GSSG levels, was analyzed during growth and development (Tables 4 and 5). To compare effectively the concentration of each cells, relative percentage values of reduced and total glutathione were calculated to the values of KAx3 cells (Figs 30 and 31). When cells were grown or starved in the absence of exogenously added GSH (*gcsA*$^{-}$ cells: 0 mM GSH; YakA^{OE}/*gcsA*$^{-}$ cells: 0 mM GSH), intracellular GSH and total glutathione were undetectable. When 1 mM GSH was added (*gcsA*$^{-}$ cells: 1 mM GSH;

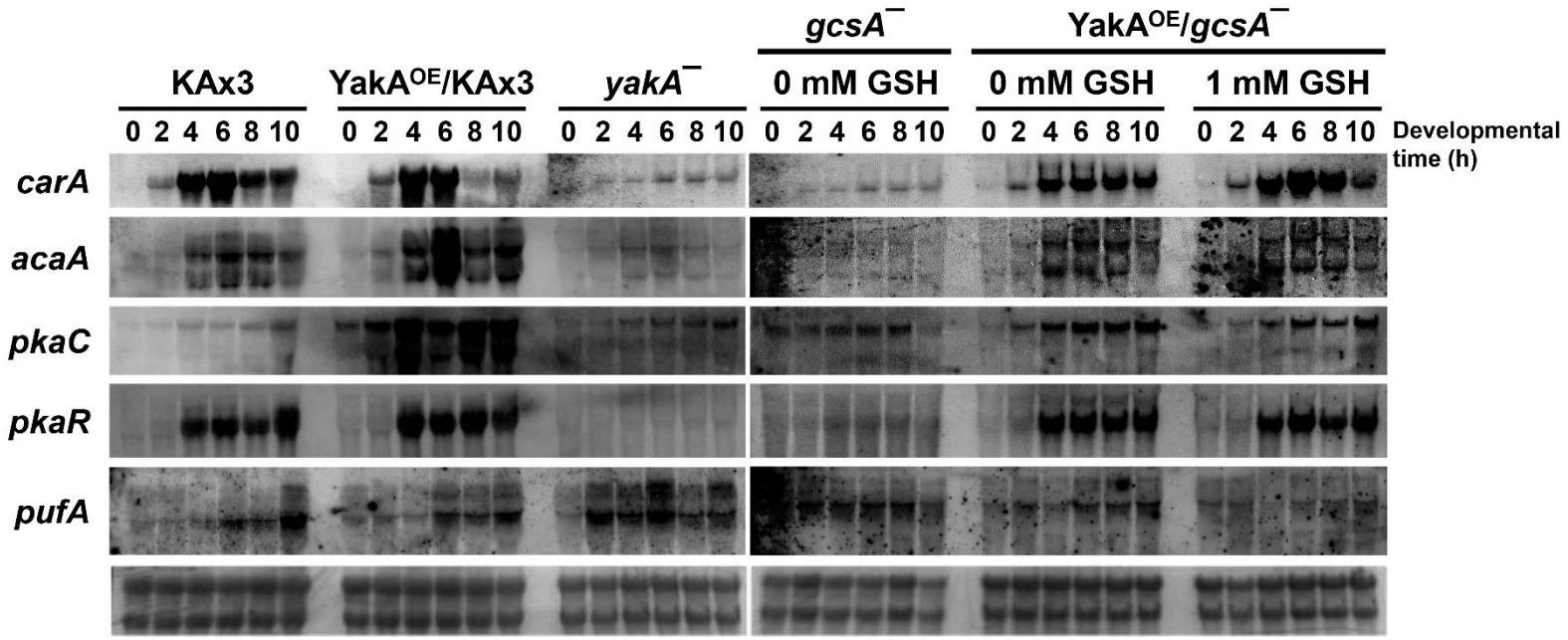


Fig. 29. Effect of YakA expression on early developmental gene expression. KAx3, YakA^{OE}/KAx3, *yakA*$^{-}$, *gcsA*$^{-}$, and YakA^{OE}/ *gcsA*$^{-}$ cells were allowed to develop in non-nutrient KK2 buffer and the expression of developmental genes was analyzed by Northern blotting. *carA*, encoding cAMP receptor 1; *acaA*, encoding adenylyl cyclase A; *pkaC*, protein kinase catalytic subunit; *pkaR*, encoding protein kinase regulatory subunit; *pufA*, encoding a RNA binding protein.

Table 4. Intracellular GSH contents of KAx3, *gcsA*$^{-}$, *yakA*$^{-}$, and YakA-expressing KAx3 and *gcsA*$^{-}$ cells[a] during growth

	GSH concentration (nmol/g[b])
KAx3	29.436 ± 2.993
YakA^{OE}/KAx3	33.606 ± 2.254
yakA^{-}	16.992 ± 2.757
gcsA^{-} 0 mM GSH	0.160 ± 0.461
gcsA^{-} 1 mM GSH	18.521 ± 2.287
YakA^{OE}/*gcsA*$^{-}$ 0 mM GSH	0.695 ± 1.389
YakA^{OE}/*gcsA*$^{-}$ 1 mM GSH	14.804 ± 2.072

[a] The values of quantitative measurements by HPLC represent mean ± S.E.M. of three independent experiments.

[b] **nmol/g**: wet weight

Table 5. Intracellular glutathione contents of KAx3, *gcsA*$^{-}$, *yakA*$^{-}$, and YakA-expressing KAx3 and *gcsA*$^{-}$ cells[a] during development

	Glutathione concentration nmol/g[b]		
	Reduced	Oxidized	Total
KAx3	26.591 ± 1.933	3.513 ± 2.668	30.105 ± 0.735
YakA^{OE}/KAx3	23.430 ± 2.148	8.602 ± 1.852	32.032 ± 0.295
yakA^{-}	10.745 ± 0.792	1.663 ± 1.915	12.408 ± 1.124
gcsA^{-} 0 mM GSH	0.418 ± 0.049	0.619 ± 0.084	1.037 ± 0.035
gcsA^{-} 1 mM GSH	2.505 ± 0.400	0.503 ± 0.865	3.007 ± 0.466
YakA^{OE}/*gcsA*$^{-}$ 0 mM GSH	0.660 ± 0.164	0.503 ± 0.120	1.163 ± 0.044
YakA^{OE}/*gcsA*$^{-}$ 1 mM GSH	2.312 ± 0.609	0.735 ± 0.936	3.047 ± 0.327

[a] The values of quantitative measurements by HPLC represent mean ± S.E.M. of three independent experiments.

[a] **nmol/g**: wet weight

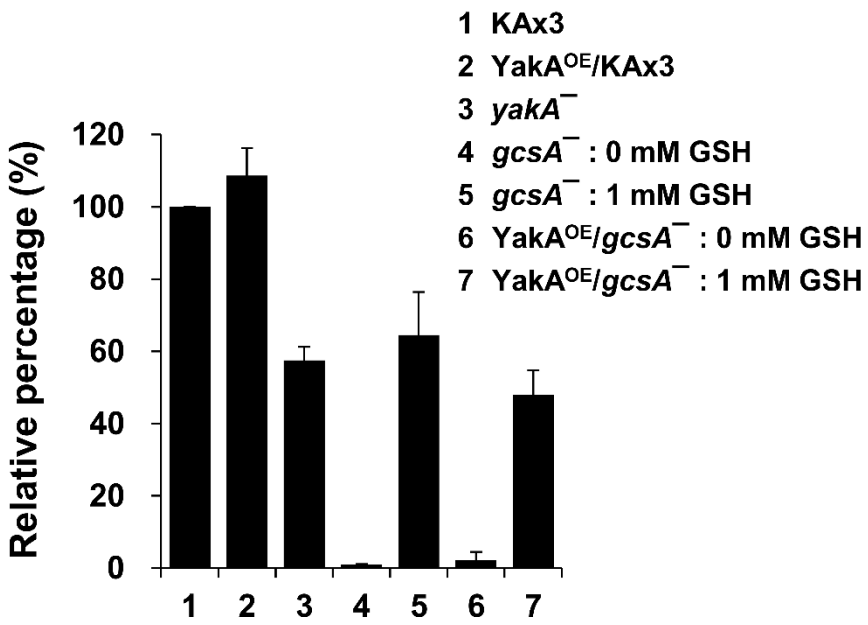
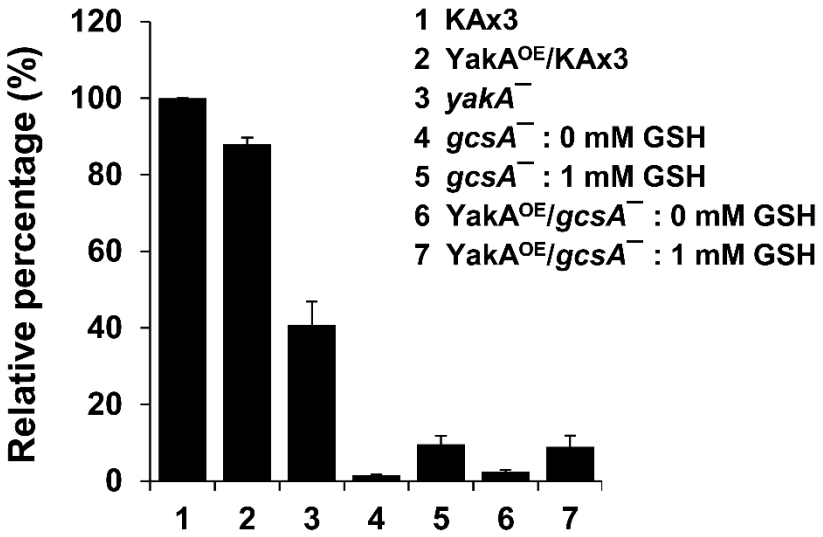


Fig. 30. Intracellular GSH contents of KAx3, *gcsA*¯, *yakA*¯, and YakA-expressing KAx3 and *gcsA*¯ cells during growth. The concentration of intracellular GSH was measured in exponentially growing KAx3, *gcsA*¯, *yakA*¯, and YakA-expressing KAx3 and *gcsA*¯ cells. The concentration was calculated in relative values compared to that of KAx3 cells. The values represent mean ± S.E.M. of three independent experiments.

A


Relative percentage (%)
120
100
80
60
40
20
0
1 2 3 4 5 6 7
1 KAx3
2 YakA^OE/KAx3
3 yakA¯
4 gcsA¯ : 0 mM GSH
5 gcsA¯ : 1 mM GSH
6 YakA^OE/gcsA¯ : 0 mM GSH
7 YakA^OE/gcsA¯ : 1 mM GSH

B

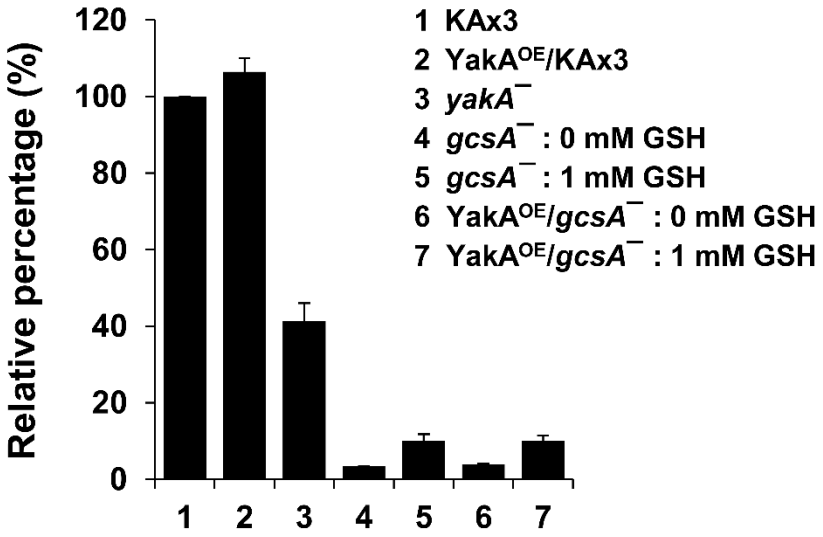

Relative percentage (%)
120
100
80
60
40
20
0
1 2 3 4 5 6 7
1 KAx3
2 YakA^OE/KAx3
3 yakA¯
4 gcsA¯ : 0 mM GSH
5 gcsA¯ : 1 mM GSH
6 YakA^OE/gcsA¯ : 0 mM GSH
7 YakA^OE/gcsA¯ : 1 mM GSH

Fig. 31. Intracellular glutathione contents of KAx3, *gcsA*$^{-}$, *yakA*$^{-}$, and YakA-expressing KAx3 and *gcsA*$^{-}$ cells during suspension development. The concentration of intracellular reduced and total glutathione was measured in KAx3, *gcsA*$^{-}$, *yakA*$^{-}$, and YakA-expressing KAx3 and *gcsA*$^{-}$ cells which were harvested at 0 h after development in suspension. The concentration of GSH (A) and the concentration of total glutathione (B), which is sum of the GSH and GSSG, were calculated in relative percentage values compared to that of KAx3 cells. The values represent mean ±S.E.M. of three independent experiments.

YakA^{OE}/*gcsA*$^{-}$ cells: 1 mM GSH) the intracellular concentration of GSH and total glutathione contents increased significantly during growth and development. And there were not significant differences between *gcsA*$^{-}$ and YakA^{OE}/*gcsA*$^{-}$ cells. In other words, the concentration of intracellular glutathione was not influenced by YakA expression in *gcsA*$^{-}$ cells. The concentration of GSH slightly increased during growth and decreased slightly during development, but the total glutathione level was higher in YakA^{OE}/KAx3 cells than that in KAx3 cells. The calculated concentration of GSSG was too low to detect and each cells showed similar levels of GSSG contents. It was interesting that YakA^{OE}/*gcsA*$^{-}$ cells proceeded to developmental life cycle although they contained intracellular GSH levels similar to those of *gcsA*$^{-}$ cells (See Discussion). These results suggest that intracellular GSH levels regulate the expression level of *yakA* but YakA does not effect on the concentration of intracellular GSH.

7. Relation between YakA and intracellular GSH

7.1. The intracellular contents of GSH in *yakA*$^{-}$ cells

To investigate the relation between GSH and YakA in detail, the concentration of intracellular GSH was measured in *yakA*$^{-}$ cells (Tables 3 and 4). In *yakA*$^{-}$ cells, the concentration of GSH and the total glutathione content were 40% and 60% lower in growing cells and in developing cells, respectively, than those in KAx3 cells (Figs 30 and 31). The results showed that intracellular GSH was significantly decreased in *yakA*$^{-}$ cells and slightly increased in YakA^{OE}/KAx3 cells. The concentration of intracellular GSH was affected by YakA expression in KAx3 cells (See Discussion).

7.2. The expression of *gcsA* in *yakA*$^{-}$ cells

Since the concentration of intracellular GSH seemed to be regulated by YakA in KAx3 cells, the expression levels of *gcsA* were examined in YakA^{OE}/KAx3 and *yakA*$^{-}$ cells. The expression of *gcsA* was difficult to detect in KAx3 cells at 0 h when cells were exposed to developmental conditions, gradually increased as cells formed aggregates, and reached a maximum at 10 h (Figs. 32 and 33). Interestingly, the level of *gcsA* mRNA was high in *yakA*$^{-}$ cells when development started (0 h). Further, the induced expression of *gcsA* mRNA was consistently maintained in *yakA*$^{-}$ cells throughout aggregation. The patterns of *gcsA* expression in KAx3 cells and YakA^{OE}/KAx3 cells were similar (Fig. 34). *yakA*$^{-}$ cells showed decreased intracellular GSH levels and increased *gcsA* expression. The levels of intracellular GSH and *gcsA* expression was not considerably changed in YakA^{OE}/*gcsA*$^{-}$ cells compared to those in KAx3 cells. These results indicate that the expression of *gcsA* is regulated transcriptionally in *yakA*$^{-}$ cells and this will be discussed more in Discussion section.

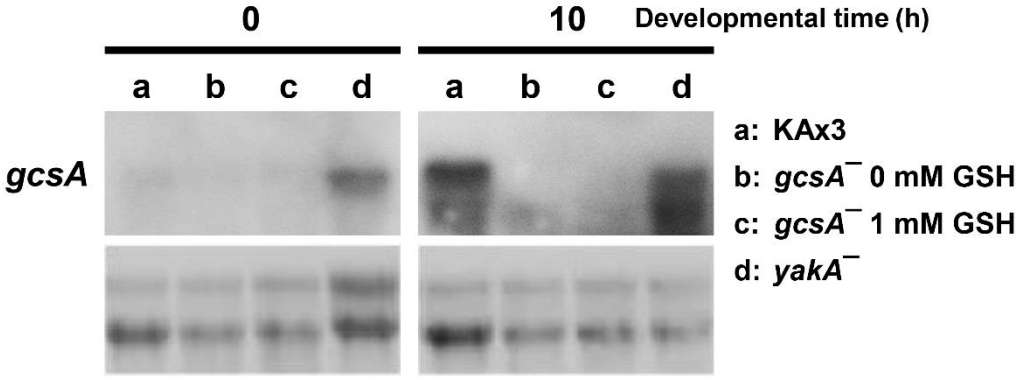


Fig. 32. Expression of *gcsA* in KAx3 and *yakA*¯ cells. KAx3, *yakA*, and *yakA*¯ cells were allowed to develop in non-nutrient KK2 buffer and total RNA was prepared at 0 h and at 10 h of development to analyze the expression *gcsA* by Northern blotting.

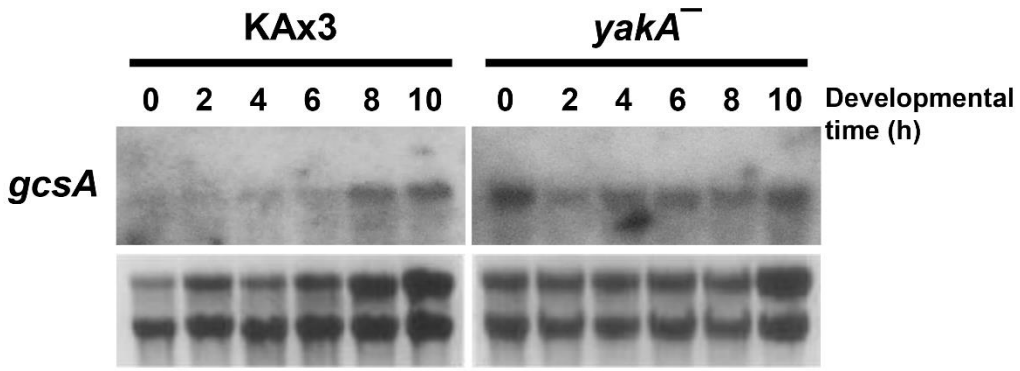


Fig. 33. Expression of *gcsA* in KAx3 and *yakA*$^{-}$ cells during aggregation progresses. KAx3 and *yakA*$^{-}$ cells were allowed to develop in non-nutrient KK2 buffer and total RNA was extracted at 2 h intervals. The expression of *gcsA* was monitored by Northern blotting analysis.

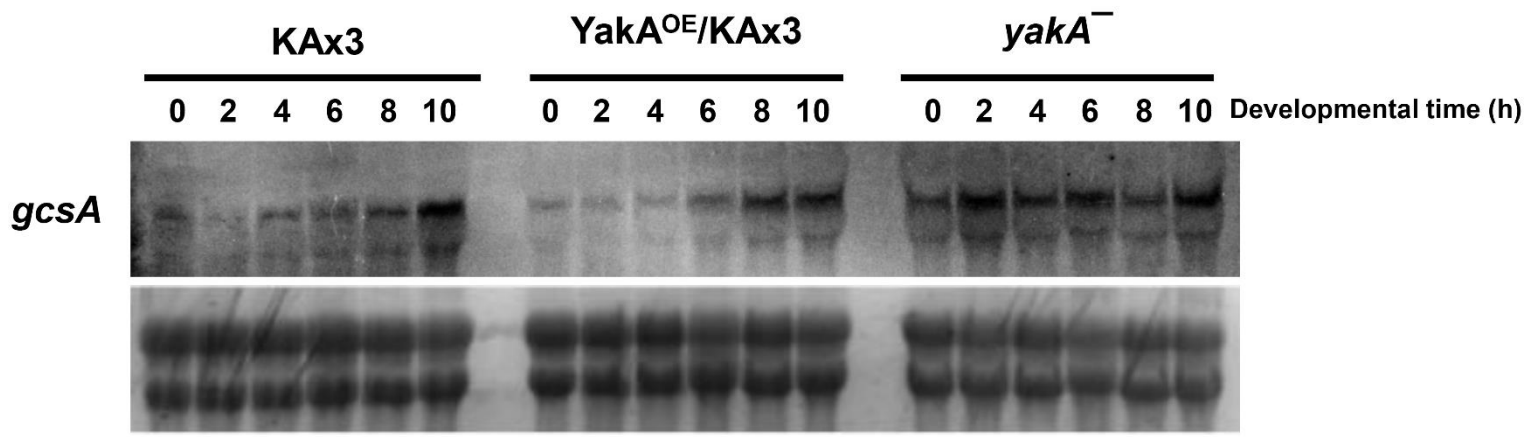


Fig. 34. Effect of YakA expression on the expression of *gcsA*. KAx3, YakA^{OE}/KAx3, and *yakA*$^{-}$ cells were allowed to develop in non-nutrient KK2 buffer and total RNA was extracted at 2 h intervals. The expression of *gcsA* was analyzed by Northern blotting.

IV. DISCUSSION

In the present study, the roles of GSH in *Dictyostelium* development were investigated by using GSH-depleted *gcsA*$^{-}$ cells defective in the synthesis of GSH. Previous reports have shown that GSH serves important roles in the normal growth and differentiation (Kim *et al.*, 2005; Choi *et al.*, 2006; Choi *et al.*, 2008). However the precise action mechanism responsible for developmental function of GSH is not unknown. According to findings in this study, intracellular GSH regulates the transition from growth to development by modulating the expression of *yakA* and its downstream regulators, which are essential for initiating development of *Dictyostelium*.

In previous research, *gcsA*$^{-}$ cells exhibit developmental defects according to the GSH concentration which is supplied in culture media before development. *gcsA*$^{-}$ cells are arrested at mound stage when pre-cultured with 0.2 mM GSH and at culmination step when pre-cultured with more than 0.5 mM GSH (Kim *et al.*, 2005). Prespore-specific genes and spore-specific genes are not expressed in *gcsA*$^{-}$ cells. In addition, the expression of *gcsA* is regulated during developmental life cycle of *Dictyostelium* (Kim *et al.*, 2005). The expression is increased during aggregation and during culmination, indicating that intracellular GSH has role in development of *Dictyostelium*. To understand the role of GSH, GSH was completely removed from *gcsA*$^{-}$ cells as described in material method. To minimize stresses of cells, the amount of added GSH was reduced gradually and incubated for 24 h in GSH-free media. As shown in Figs. 5 and 6, GSH-depleted *gcsA*$^{-}$ cells did not aggregate and also did not show chemotactical movements. *Dictyostelium* could not develop without GSH.

In general, GSH is known to be an essential metabolite and a major antioxidant in most eukaryotic cells. Disruption of GSH biosynthesis results in GSH auxotroph (Grant *et al.*, 1996; Chaudhuri *et al.*, 1997; Baek *et al.*, 2004)

and cell death through apoptosis (Hall, 1999; Madeo *et al.*, 1999; Baek *et al.*, 2004) in other organisms. In *Dictyostelium*, depletion of GSH by disrupting *gcsA* encoding GCS, the first enzyme in GSH biosynthesis, also caused GSH auxotroph but apoptosis was not induced. *gcsA*-null cells survived in rich media or in salt-only minimal media, though they showed cell cycle arrest or developmental defects. It was reasoned that the defects in development caused by GSH depletion may result from oxidative stress. Exogenous thiol compounds have been supplemented to compensate the role of GSH. NAC and DTT are cell permeable and stabilize the cellular reducing potential in other organisms (Abello *et al.*, 1994) and have protective role against oxidative stress (McGowan *et al.*, 1996; Reid *et al.*, 2005). DTT rescued the defects caused by oxidative stress by GSH depletion (Grant *et al.*, 1996). NAC also rescued apoptosis caused by GSH depletion (Shi *et al.*, 1994). It was found in this study that GSH, but not exogenous DTT and NAC rescued the developmental defect of the GSH-depleted *gcsA*$^{-}$ cells (Fig. 8). In addition, *gcsA*$^{-}$ cells did not develop with the addition of a general antioxidant, ascorbic acid. However, the oxidized form of GSH (GSSG) and the precursor of GSH (γ-GC), which can be converted into GSH, supported normal development of *gcsA*$^{-}$ cells, though it was not as much as GSH did. These results suggest that essential requirement of GSH in *Dictyostelium* development is probably not related to its redox properties. GSH itself has indispensable roles that cannot be compensated for by antioxidant.

The transition from growth to development is regulated by a complex series of signals designed to ensure that aggregation occurs under optimal conditions, especially through regulating gene expression. To understand the roles of GSH in development of *Dictyostelium*, the expression levels of several

genes which are required to initiate *Dictyostelium* development was determined. In absence of GSH, the expression of these genes was not precisely controlled in *gcsA*$^{-}$ cells. For example, *gcsA*$^{-}$ cells failed to decrease the expression of a vegetative-stage specific serine proteinase, *cprD* (Fig. 9), and the expression of *dscA* and *dia2* decreased in the GSH-depleted *gcsA*$^{-}$ cells (Fig. 10). The expression of *cprD*, *dscA*, and *dia2* act as markers for the transition from growth to development. *cprD* is expressed extensively during growth, but not during development. Members of the discoidin I gene family are among the first to be activated by prestarvation responses (Clarke *et al.*, 1987). During growth, cells secrete prestarvation factor (PSF) and estimate their density relative to the concentration of nutrients. When PSF reaches an appropriate concentration, it induces the expression of *dscA*, which encodes the discoidin I alpha chain, and prepares cells for developmental initiation. Discoidin accumulates continuously during early development until its transcription is inhibited by extracellular cAMP at the end of the aggregation phase. Thus, the expression of discoidin is an excellent indicator of the cell state in the developmental life cycle. *dia2* (differentiation-associated protein) transcripts accumulate exclusively in differentiating cells, but they are not detected in the growing cells (Chae *et al.*, 1998; Hirata *et al.*, 2008; Maeda, 2005). Interestingly, *dscA* and *dia2* were not expressed in GSH-depleted *gcsA*$^{-}$ cells (Fig. 10). However, exogenous addition of 1 mM GSH induced *dscA* and *dia2* expression. The expression of *cprD*, *dscA*, and *dia2* indicates that *Dictyostelium* cells did not initiate developmental cycle without GSH even in the presence of a developmental signal.

The early events in multicellular development of *Dictyostelium*, in particular the role of the cAMP signaling pathway, have been extensively studied (Loomis, 1998). cAMP signaling plays a central role in control of

multicellular aggregate formation. For the initiation of development, the expression of ACA and cAR1 is sophisticatedly regulated. The results analyzed in this study demonstrated that the expression of *carA* and *acaA* was not enhanced in the absence of GSH in *gcsA*$^{-}$ cells (Fig. 9). Failure of the induction of *carA* and *acaA* expression in *gcsA*$^{-}$ cells suggests a defect in the activation of cAMP-dependent signaling. Some groups reported that administering pulses of exogenous cAMP rescues the expression of *carA* and other components of cAMP signaling in some aggregation-defective mutants such as *gα3*$^{-}$ and Ddmyb2^{-} cells (Khosla *et al.*, 1996; Brandon and Podgorski, 1997; Otsuka and Van Haastert, 1998). However, pulsed addition of exogenous cAMP pulses did not rescue the expression of *carA* and *acaA* or developmental defect in *gcsA*$^{-}$ cells in the absence of GSH (Figs. 11 and 12). Therefore, it was suspected that g*csA*$^{-}$ cells might not response to exogenously added cAMP because of their lack of extracellular cAMP recognition. However, cAR1 expression also failed to produce aggregates. In the cAMP signaling system, the expression of ACA, cAR1, and Gα2 are regulated by positive feedback loop and enlarge cAMP pulses (Klein *et al.*, 1998; Pitt *et al.*, 1992; Kumagai *et al.*, 1989). Interestingly, the components of cAMP signaling pathway such as *carA*, *acaA*, and *gpaB* were expressed in sufficient amount to transmit cAMP signals in *gcsA*$^{-}$ cells in the absence of GSH (Fig. 15). These data demonstrate that lack of cAMP oscillations, the synthesis and recognition of cAMP, are not a main cause of the developmental defect in *gcsA*$^{-}$ cells. Thus, GSH may be required at a step upstream of cAMP signaling. Taken together, these findings strongly suggest that intracellular GSH plays essential roles in regulation of the transition from growth to development in *Dictyostelium*.

Next, the YakA signaling pathway was considered, which is known the earliest development regulating system prior to cAMP signaling in response to starvation signal. Previous reports have shown that YakA is necessary for the transition from growth to development in *Dictyostelium* (Souza *et al.*, 1998) and that the expression of YakA is required for the turning off growth-phase genes and for the induction of differentiation-associated genes. Moreover, *yakA*$^{-}$ cells show similar developmental phenotype and gene expression patterns to those of GSH-depleted *gcsA*$^{-}$ cells. *yakA*$^{-}$ cells and GSH-depleted *gcsA*$^{-}$ cells did not initiate development (Fig. 24) and showed undetectably low *carA* and *acaA* expression (Fig. 25). Surprisingly, the mRNA level of *yakA* significantly decreased in GSH-depleted *gcsA*$^{-}$ cells (Fig. 16). Further, the expression of *yakA* was modulated by the concentration of GSH added exogenously in KAx3 and *gcsA*$^{-}$ cells (Figs. 16 and 17). Therefore, these findings provide compelling evidence to support clearly the conclusion that GSH regulates the initiation of development by inducing the expression of *yakA*.

The role of GSH in regulating the expression of *yakA* was supported by the results acquired from monitoring other components of YakA pathway. As expected, similar to *yakA*$^{-}$ cells, GSH-depleted *gcsA*$^{-}$ cells showed increased *pufA* expression (Figs. 22 and 25) and decreased PKA activity (Fig. 23). Thus, it is apparent that the depletion of GSH blocks development because the YakA signaling system is not activated in the absence of GSH.

PKA activity was clearly regulated by the availability of intracellular GSH. The expression of *pkaC* and *pkaR* was also determined in *gcsA*$^{-}$ and *yakA*$^{-}$ cells. *pkaC* was transcribed normally as in KAx3 cells regardless of the presence of intracellular GSH or the expression of *yakA* (Figs. 22 and 25). In contrast, the expression of *pkaR* was unpredictable in *gcsA*$^{-}$ and *yakA*$^{-}$ cells. Increased

expression of *pkaR* was expected from the decreased PKA activity in GSH-depleted *gcsA*$^{-}$ cells and *yakA*$^{-}$ cells. However, the expression of *pkaR* was inhibited in both *gcsA*$^{-}$ and *yakA*$^{-}$ cells (Fig. 25) and further decreases was induced by the addition of GSH in both *gcsA*$^{-}$ and cAR1^{OE}/*gcsA*$^{-}$ cells (Figs. 15 and 22). YakA expression in KAx3 (YakA^{OE}/KAx3) and *gcsA*$^{-}$ (YakA^{OE}/*gcsA*$^{-}$) cells showed the increased expression of *pkaR* (Fig. 29). The analysis of Northern blotting showed that the transcriptional expression of *pkaR* was inhibited by GSH and induced by YakA. These findings suppose that the expression of *pkaR* is regulated in different way with the expression of *pkaC* and the transcriptional regulation of *pkaR* is not a critical factor to determine the activity of PKA.

To confirm the relationship between GSH and YakA, YakA was constitutively expressed in KAx3 and *gcsA*$^{-}$ cells (YakA^{OE}/KAx3 and YakA^{OE}/*gcsA*$^{-}$). GSH-depleted *gcsA*$^{-}$ cells developed and formed aggregates when YakA was expressed (Figs. 27 and 28). Moreover, the expression of developmental genes such as *carA*, *acaA*, and *pkaC* were increased by the constitutive expression of YakA in *gcsA*$^{-}$ cells (Fig. 29). These data imply that the developmental defects of GSH-depleted *gcsA*$^{-}$ cells are caused by the decreased expression of *yakA* and intracellular GSH induces the expression of *yakA* in response to a starvation signal to initiate developmental processes. Taken together, these findings suggest that intracellular GSH regulates the transition from growth to development by modulating YakA and downstream signaling.

The concentration of intracellular GSH in *yakA*$^{-}$ cells decreased by approximately 40% compared with KAx3 cells during growth and development (Figs. 30 and 31), although *gcsA* was constitutively expressed during growth

and aggregation processes (Figs. 32 and 33). In contrast, there were not significant differences of *gcsA* expression levels and intracellular GSH contents between in YakA^{OE}/KAx3 and KAx3 cells, indicating that YakA does not directly regulate intracellular GSH levels. According to Bloomfield and Pears (2003), a significant amount of superoxide is generated in response to CMF during the transition to the multicellular phase of development. Further, Taminato *et al.* (2002) reported that *yakA*$^{-}$ cells are hypersensitive to oxidative and nitrosoative stress. Therefore, it was postulated that the hypersensitive reaction to oxidative stress may cause the decrease in the intracellular GSH level in *yakA*$^{-}$ cells. Further, *yakA*$^{-}$ cells may consume more GSH than KAx3 cells to protect against oxidative stress and thus low concentration of intracellular GSH was detected. Increased *gcsA* expression could be explained by the decreased intracellular GSH level in *yakA*$^{-}$ cells. The decreased intracellular GSH level may induce *gcsA* expression through feedback regulation.

Furthermore, the intracellular content of GSH decreased by 90% in *gcsA*$^{-}$ cells which were developed with the addition of 1mM GSH compared to KAx3 cells when development commenced but at 10 h after development, the concentration rose to 40% of that in KAx3 cells (Fig. 7). In growing *gcsA*$^{-}$ cells, the GSH content was higher than that in developing cells but reached a limit of 50–60% of the level in KAx3 cells (Fig. 3), suggesting that exogenously added GSH is not completely incorporated by cells and that GSH enters *gcsA*$^{-}$ cells in proportion to the time of exposure. These results could explain the delayed developmental process and transcriptional expression of early developmental genes in *gcsA*$^{-}$ cells which were developed with 1 mM GSH. Exogenously added GSH did not cross the plasma membrane completely and decreased intracellular GSH compare to KAx3 cells result in incomplete restoration of

developmental defect. Remarkably, a very low level of GSH can induce the development of *gcsA*$^{-}$ cells, suggesting that intracellular GSH plays a vital role in *Dictyostelium* development.

Finally, a model of developmental initiation in which GSH regulates the expression of YakA and other components of the YakA signaling pathway is proposed (Fig. 35). GSH plays an essential role in the transition from growth to development by regulating YakA and signal transduction when development initiates. It is expected that further studies designed to elucidate the molecular mechanisms that govern the regulation of gene transcription by GSH may provide insight into general mechanisms underlying initiation of cell development.

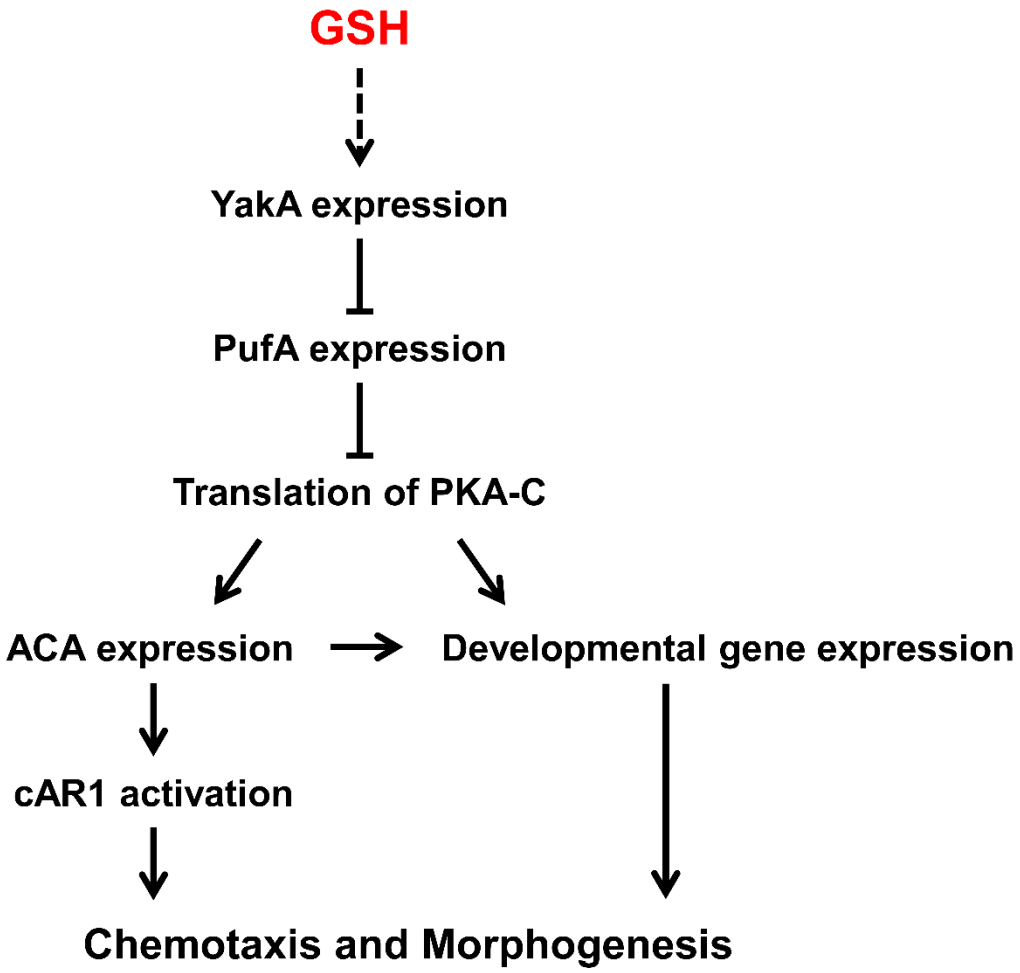


Fig. 35. Regulation of developmental initiation by intracellular GSH in *Dictyostelium discoideum*.

V. REFERENCES

Abe, K., and Yanagisawa, K. (1983) A new class of rapidly developing mutants in *Dictyostelium discoideum*: implications for cyclic AMP metabolism and cell differentiation. *Dev. Biol.* **95**, 200-210.

Abello, P.A., Fidler, S.A., and Buchman, T.G. (1994) Thiol reducing agents modulate induced apoptosis in porcine endothelial cells. *Shock* **2**, 79-83.

Akerboom, T. P., Bilzer, M., and Sies, H. (1982) The relationship of biliary glutathione disulfide efflux and intracellular glutathione disulfide content in perfused rat liver. *J. Biol. Chem.* **257**, 4248-4252.

Allen, R. G., Newton, R. K., Sohal, R. S., Shipley, G. L., and Nations, C. (1985) Alterations in superoxide dismutase, glutathione, and peroxides in the plasmodial slime mold *Physarum polycephalum* during differentiation. *J. Cell Physiol.* **125**, 413-419.

Anjard, C., Etchebehere, L., Pinaud, S., Veron, M., and Reymond, C. D. (1993) An unusual catalytic subunit for the cAMP-dependent protein kinase of *Dictyostelium discoideum*. *Biochemistry* **32**, 9532-9538.

Anjard, C., Pinaud, S., Kay, R. R., and Reymond, C. D. (1992) Overexpression of Dd PK2 protein kinase causes rapid development and affects the intracellular cAMP pathway of *Dictyostelium discoideum*. *Development* **115**, 785-790.

Aw, T. Y. (2003) Cellular Redox: A modulator of intestinal epithelial cell proliferation. *Physiology* **18**, 201-204.

Baek, Y. U., Kim, Y. R., Yim, H. S., and Kang, S. O. (2004) Disruption of gammaglutamylcysteine synthetase results in absolute glutathione auxotrophy and apoptosis in *Candida albicans*. *FEBS Lett.* **556**, 47-52.

Bella, D. L., Hirschberger, L. L., Hosokawa, Y., and Stipanuk, M. H. (1999) Mechanisms involved in the regulation of key enzymes of cysteine metabolism in rat liver *in vivo*. *Am. J. Physiol.* **276**, E326-335.

Bloomfield, G., and Pears, C. (2003) Superoxide signalling required for multicellular development of *Dictyostelium*. *J. Cell Sci.* **16**, 3387-3397.

Brandon, M. A., and Podgorski, G. J. (1997) G alpha 3 regulates the cAMP signaling system in *Dictyostelium*. *Mol. Biol. Cell* **8**, 1677-1685.

Cairns, N. G., Pasternak, M., Wachter, A., Cobbett, C. S., and Meyer, A. J. (2006) Maturation of *Arabidopsis* seeds is dependent on glutathione biosynthesis within the embryo. *Plant Physiol.* **141**, 446-455.

Chae, S. C., Inazu, Y., Amagai, A., and Maeda, Y. (1998) Underexpression of a novel gene, *dia2*, impairs the transition of *Dictyostelium* cells from growth to differentiation. *Biochem. Biophys. Res. Commun.* **252**, 278-283.

Chaudhuri, B., Ingavale, S., and Bachhawat, A. K. (1997) *apd1+*, a gene required for red pigment formation in *ade6* mutants of *Schizosaccharomyces pombe*, encodes an enzyme required for glutathione biosynthesis: a role for glutathione and a glutathione-conjugate pump. *Genetics* **145**, 75-83.

Chen, M. Y., Long, Y., and Devreotes, P. N. (1997) A novel cytosolic regulator, Pianissimo, is required for chemoattractant receptor and G protein-mediated activation of the 12 transmembrane domain adenylyl cyclase in *Dictyostelium*. *Genes Dev.* **11**, 3218-3231.

Choi, C. H., Kim, B. J., Jeong, S. Y., Lee, C. H., Kim, J. S., Park, S. J., Yim, H. S. and Kang, S. O. (2006) Reduced glutathione levels affect the culmination and cell fate decision in *Dictyostelium discoideum*. *Dev. Biol.* **295**, 523-533.

Choi, C. H., Park, S. J., Jeong, S. Y., Yim, H. S., and Kang, S. O. (2008) Methylglyoxal accumulation by glutathione depletion leads to cell cycle arrest in *Dictyostelium*. *Mol. Microbiol.* **70**, 1293-1304.

Clarke, M., Kayman, S. C., and Riley, K. (1987) Density-dependent induction of discoidin-I synthesis in exponentially growing cells of *Dictyostelium discoideum*. *Differentiation* **34**, 79-87.

Clarke, M., Yang, J., and Kayman, S. C. (1988) Analysis of the prestarvation response in growing cells of *Dictyostelium discoideum*. *Dev. Genet.* **9**, 315-326.

Cocucci, S.M. and Sussman, M. (1970) RNA in cytoplasmic and nuclear fractions of cellular slime mold amebas. *J. Cell Sci.* **45**, 399-407.

Cotter, D. A., Dunbar, A. J., Buconjic, S. D., and Wheldrake, J. F. (1999) Ammonium phosphate in sori of *Dictyostelium discoideum* promotes spore dormancy through stimulation of the osmosensor ACG. *Microbiology* **145**, 1891-1901.

de Gunzburg, J., Franke, J., Kessin, R. H., and Veron, M. (1986) Detection and developmental regulation of the mRNA for the regulatory subunit of the cAMP-dependent protein kinase of *D. discoideum* by cell-free translation. *EMBO J.* **5**, 363-367.

Devreotes, P. N. (1983) Cyclic nucleotides and cell-cell communication in Dictyostelium discoideum. Advances in cyclic nucleotide research.

Devreotes, P.N. (1994) G protein-linked signaling pathways control the developmental program of *Dictyostelium*. *Neuron* **12**, 235-241.

Devreotes, P. N., and Zigmond, S. H. (1988) Chemotaxis in eukaryotic cells: a focus on leukocytes and *Dictyostelium*. *Annu. Rev. Cell Biol.* **4**, 649-686.

Dinauer, M. C., Mackay, S. A., and Devreotes, P. N. (1980) Cyclic 3', 5'-AMP relay in *Dictyostelium discoideum* III. The relationship of cAMP synthesis and secretion during the cAMP signaling response. *J. Cell Biol.* **86**, 537-544.

Dumollard, R., Ward, Z., Carroll, J., and Duchen, M. R. (2007) Regulation of redox metabolism in the mouse oocyte and embryo. *Development* **134**, 455-465.

Durston, A. J. (1976) Tip formation is regulated by an inhibitory gradient in the *Dictyostelium discoideum* slug. *Nature* London: **263**, 126-129.

Eichinger, L., Pachebat, J. A., Glöckner, G., Rajandream, M. A., Sucgang, R., Berriman, M., Song, J., Olsen, R., Szafranski, K., Xu, Q., Tunggal, B.,

Kummerfeld, S., Madera, M., Konfortov, B. A., Rivero, F., Bankier, A. T., Lehmann, R., Hamlin, N., Davies, R., Gaudet, P., Fey, P., Pilcher, K., Chen, G., Saunders, D., Sodergren, E., Davis, P., Kerhornou, A., Nie, X., Hall, N., Anjard, C., Hemphill, L., Bason, N., Farbrother, P., Desany, B., Just, E., Morio, T., Rost, R., Churcher, C., Cooper, J., Haydock, S., van Driessche, N., Cronin, A., Goodhead, I., Muzny, D., Mourier, T., Pain, A., Lu, M., Harper, D., Lindsay, R., Hauser, H., James, K., Quiles, M., Madan Babu, M., Saito, T., Buchrieser, C., Wardroper, A., Felder, M., Thangavelu, M., Johnson, D., Knights, A., Loulseged, H., Mungall, K., Oliver, K., Price, C., Quail, M. A., Urushihara, H., Hernandez, J., Rabbinowitsch, E., Steffen, D., Sanders, M., Ma, J., Kohara, Y., Sharp, S., Simmonds, M., Spiegler, S., Tivey, A., Sugano, S., White, B., Walker, D., Woodward, J., Winckler, T., Tanaka, Y., Shaulsky, G., Schleicher, M., Weinstock, G., Rosenthal, A., Cox, E. C., Chisholm, R. L., Gibbs, R., Loomis, W. F., Platzer, M., Kay, R. R., Williams, J., Dear, P. H., Noegel, A. A., Barrell, B., and Kuspa, A. (2005) The genome of the social amoeba *Dictyostelium discoideum*. *Nature* **435**, 43-57.

Fang, Y. Z., Yang, S., and Wu, G. (2002) Free radicals, antioxidants, and nutrition. *Nutrition* **18**, 872-879.

Feinberg, A. P., and Vogelstein, B. (1983) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132**, 6-13.

Firtel, R. A. (1995) Integration of signaling information in controlling cell fate decisions in *Dictyostelium. Genes Dev.* **9**, 1427-1444.

Forbes, A., and Lehmann, R. (1998) Nanos and Pumilio have critical roles in the development and function of *Drosophila* germline stem cells. *Development* **125**, 679-690.

Franklin, C. C., Rosenfeld-Franklin, M. E., White, C., Kavanagh, T. J., and Fausto, N. (2003) TGFβ1-induced suppression of glutathione antioxidant defenses in hepatocytes: caspase-dependent post-translational and caspase-independent transcriptional regulatory mechanisms. *FASEB J.* **17**, 1535-1537.

Galardi-Castilla, M., Pergolizzi, B., Bloomfield, G., Skelton, J., Ivens, A., Kay, R. R., Bozzaro, S., and Sastre, L. (2008) SrfB, a member of the Serum Response Factor family of transcription factors, regulates starvation response and early development in *Dictyostelium*. *Dev. Biol.* **316**, 260-274.

Galter, D., Mihm, S., and Droge, W. (1994) Distinct effects of glutathione disulphide on the nuclear transcription factor kappa B and the activator protein-1. *Eur. J. Biochem.* **221**, 639-648.

Garcia-Ruiz, C., and Fernandez-Checa, J. C. (2006) Mitochondrial glutathione: hepatocellular survival-death switch. *J. Gastroenterol. Hepatol.* (*Suppl*) **21**, S3-S6.

Garcia-Ruiz, C., and Fernández-Checa, J. C. (2007) Redox regulation of hepatocyte apoptosis. *J. Gastroenterol. Hepatol.* (*Suppl*) **22**, S38-S42.

Gardiner, C. S., and Reed, D. J. (1994) Status of glutathione during oxidant-induced oxidative stress in the preimplantation mouse embryo. *Biol.Reprod.* **51**, 1307-1314.

Gomer, R. H., Yuen, I. S., and Firtel, R. A. (1991) A secreted 80 x 10(3) Mr protein mediates sensing of cell density and the onset of development in *Dictyostelium***.** *Development* **112**, 269-278.

Gomi, A., Masuzawa, T., Ishikawa, T., and Kuo, M. T. (1997) Posttranscriptional regulation of MRP/GS-X pump and γ-glutamylcysteine synthetase expression by 1-(4-amino-2-methyl-5-pyrimidinyl)-methyl-3-(2-chloroethyl)-3-nitrosourea and by cycloheximide in human glioma cells. *Biochem. Biophys. Res. Commun.* **239**, 51-56.

Grant, C. M., MacIver, F. H., and Dawes, I. W. (1996) Glutathione is an essential metabolite required for resistance to oxidative stress in the yeast *Saccharomyces cerevisiae*. *Curr. Genet.* **29**, 511–515.

Grant, C. M., MacIver, F. H., and Dawes, I. W. (1997) Glutathione synthetase is dispensable for growth under both normal and oxidative stress conditions in the yeast *Saccharomyces cerevisiae* due to an accumulation of the dipeptide gamma-glutamylcysteine. *Mol. Biol. Cell* **8**, 1699-1707.

Griffith, O. W., and Mulcahy, R. T. (1999) The enzymes of glutathione synthesis: γ-glutamylcysteine synthetase. *Adv. Enzymol. Relat. Areas Mol. Biol.* **73**, 209-267.

Grinberg, L., Fibach, E., Amer, J., and Atlas, D. (2005) *N*-acetylcysteine amide, a novel cell-permeating thiol, restores cellular glutathione and protects human red blood cells from oxidative stress, *Free Radical Biol. Med.* **38**, 136-145.

Gross, J. D. (1994) Developmental decisions in *Dictyostelium discoideum*. *Microbiol. Rev.* **58**, 330-351.

Hall, A. G. (1999) The role of glutathione in the regulation of apoptosis. *Eur. J. Clin. Invest.* **29**, 238-245.

Halliwell, B., and Gutteridge, J. M. C. (1989) Free Radicals in Biology and Medicine. London: Oxford Univ. Press.

Harwood, A. J., Hopper, N. A., Simon, M. N., Bouzid, S., Veron, M., and Williams, J. G. (1992) Multiple roles for cAMP-dependent protein kinase during *Dictyostelium* development. Dev. Biol. 149, 90-99.

Hayes, J. D., and McLellan, L. I. (1999) Glutathione and glutathione-dependent enzymes represent a co-ordinately regulated defence against oxidative stress. *Free Radic. Res.* **31**, 273-300.

Hirata, K., Amagai, A., Chae, S. C., Hirose, S., and Maeda, Y. (2008) Involvements of a novel protein, DIA2, in cAMP signaling and spore differentiation during *Dictyostelium* development. *Differentiation* **76**, 310-322.

Hopper, N. A., Anjard, C., Reymond, C. D., and Williams, J. G. (1993a) Induction of terminal differentiation of *Dictyostelium* by cAMP-dependent protein kinase and opposing effects of intracellular and extracellular cAMP on stalk cell differentiation. *Development* 119, 147-154.

Hwang, C., Sinskey, A. J., and Lodish, H. F. (1992) Oxidized redox state of glutathione in the endoplasmic reticulum. *Science* **257**, 1496-1502.

Iijima, N., Takagi, T., and Maeda, Y. (1995) A Proteinous Factor Mediating Intercellular Communication during the Transition of *Dictyostelium* Cells from Growth to Differentiation. *Zool. Sci.* **12**, 61-69.

Insall, R., Kuspa, A., Lilly, P. J., Shaulsky, G., Levin, L. R., Loomis, W. F., and Devreotes, P. (1994) CRAC, a cytosolic protein containing a pleckstrin homology domain, is required for receptor and G protein-mediated activation of adenylyl cyclase in *Dictyostelium*. *J. Cell Biol.* **126**, 1537-1545.

Jacquet, M., Guilbaud, R., and Garreau, H. (1988) Sequence analysis of the DdPYR5-6 gene coding for UMP synthase in *Dictyostelium discoideum* and comparison with orotate phosphoribosyl transferases and OMP decarboxylases. *Mol. Gen. Genet.* **211**, 441-445.

Johnson, R. L., Saxe, C. 3rd., Gollop, R., Kimmel, A. R., and Devreotes, P. N. (1993) Identification and targeted gene disruption of cAR3, a cAMP receptor subtype expressed during multicellular stages of *Dictyostelium* development. *Genes Dev.* **7**, 273-282.

Johnson, R. L., Vaughan, R. A., Caterina, M. J., Van Haastert, P. J., and Devreotes, P. N. (1991) Overexpression of the cAMP receptor 1 in growing *Dictyostelium* cells. *Biochemistry* **30**, 6982-6986.

Kaplowitz, N., Aw, T. Y., and Ookhtens, M. (1985) The regulation of hepatic glutathione. *Annu. Rev. Pharmacol. Toxicol.* **25**, 715-744.

Katoh, M., Chen, G., Roberge, E., Shaulsky, G., and Kuspa, A. (2007) Developmental commitment in *Dictyostelium discoideum*. *Eukaryotic cell* **6**, 2038-2045.

Kawamura, N. (1960) Cytochemical and quantitative study of protein-bound sulfhydryl and disulfide groups in eggs of *Arbacia* during the first cleavage. *Exp. Cell Res.* **20**, 127-138.

Ken, R., and Singleton, C. K. (1994) Redundant regulatory elements account for the developmental control of a ribosomal protein gene of *Dictyostelium discoideum*. *Differentiation* **55**, 97-103.

Ketterer, B., Coles, B., and Meyer, D. J. (1983) The role of glutathione in detoxication. *Environ. Health Perspect.* **49**, 59-69.

Kesbeke, F, Snaar-Jagalska, B. E., and Van Haastert, P. J. (1988) Signal transduction in *Dictyostelium fgd* A mutants with a defective interaction between surface cAMP receptors and a GTP-binding regulatory protein. *J. Cell Biol.* **107**, 521-528.

Khosla, M., Spiegelman, G. B., and Weeks, G. (1996) Overexpression of an activated *rasG* gene during growth blocks the initiation of *Dictyostelium* development. *Mol. Cell Biol.* **16**, 4156-4162.

Kim, B. J., Choi, C. H., Lee, C. H., Jeong, S. Y., Kim, J. S., Kim, B. Y., Yim, H. S. and Kang, S. O. (2005) Glutathione is required for growth and prespore cell differentiation in *Dictyostelium*. *Dev. Biol.* **284**, 387-398.

Kim, J. S., Seo, J. H., Yim, H. S., and Kang, S. O. (2011) Homeoprotein Hbx4 represses the expression of the adhesion molecule DdCAD-1 governing cytokinesis and development. *FEBS Lett.* **585**, 1864-1872.

Klein, P. S., Sun, T. J., Saxe, C. L. 3rd., Kimmel, A. R., Johnson, R. L., and Devreotes, P. N. (1988) A chemoattractant receptor controls development in *Dictyostelium discoideum*. *Science* **241**, 1467-1472.

Klein, P., Theibert, A., and Devreotes, P. (1988) Identification and ligand-induced modification of the cAMP receptor in *Dictyostelium*. *Methods Enzymol.* **159**, 267-278.

Kriebel, P. W., Barr, V. A., and Parent, C. A. (2003) Adenylyl cyclase localization regulates streaming during chemotaxis. *Cell* **112**, 549-560.

Kumagai, A., Pupillo, M., Gundersen, R., Miake-Lye, R., Devreotes, P. N., and Firtel, R. A. (1989) Regulation and function of G alpha protein subunits in *Dictyostelium*. *Cell* **57**, 265-275.

Lacombe, M. L., Podgorski, G. J., Franke, J., and Kessin, R. H. (1986) Molecular cloning and developmental expression of the cyclic nucleotide phosphodiesterase gene of *Dictyostelium discoideum*. *J. Biol. Chem.* **261**, 16811-16817.

Levi, S., Polyakov, M., and Egelhoff, T. T. (2000) Green fluorescent protein and epitope tag fusion vectors for *Dictyostelium discoideum*. *Plasmid* **44**, 231-238.

Lewin, S. (1976) Vitamin C: Its molecular biology and medical potential. New York, NY: Academic press 42-59.

Lilly, P., Wu, L. I. J. U. N., Welker, D. L., and Devreotes, P. N. (1993) A G-protein beta-subunit is essential for *Dictyostelium development*. *Genes Dev.* **7**, 986-995.

Lilly, P. J., and Devreotes, P. N. (1994) Identification of CRAC, a cytosolic regulator required for guanine nucleotide stimulation of adenylyl cyclase in *Dictyostelium*. *J. Biol. Chem.* **269**, 14123-14129.

Lilly, P. J., and Devreotes, P. N. (1995) Chemoattractant and GTP gamma S-mediated stimulation of adenylyl cyclase in *Dictyostelium* requires translocation of CRAC to membranes. *J. Cell Biol.* **129**, 1659-1665.

Lindermayr, C., Sell, S., Müller, B., Leister, D., and Durner, J. (2010) Redox regulation of the NPR1-TGA1 system of Arabidopsis thaliana by nitric oxide. *Plant Cell* **22**, 2894-2907.

Loomis, W. F. (1982) The spatial pattern of cell-type differentiation in *Dictyostelium*. *Dev. Biol.* **93**, 279-284.

Loomis, W. F. (1998) Role of PKA in the timing of developmental events in *Dictyostelium* cells. *Microbiol. Mol. Biol. Rev.* **62**, 684-694.

Louis, J. M., Ginsburg, G. T., and Kimmel, A. R. (1994) The cAMP receptor CAR4 regulates axial patterning and cellular differentiation during late development of *Dictyostelium*. *Genes Dev.* **8**, 2086-2096.

Louis, J. M., Saxe, C. L. 3rd., and Kimmel, A. R. (1993) Two transmembrane signaling mechanisms control expression of the cAMP receptor gene CAR1 during *Dictyostelium* development. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 5969-5973.

Lu, S. C. (1999) Regulation of hepatic glutathione synthesis: current concepts and controversies. *FASEB J.* **13**, 1169-1183.

Lu, S. C. (2000) Regulation of glutathione synthesis. *Curr. Top. Cell Regul.* **36**, 95-116.

Lu, S. C. (2009) Regulation of glutathione synthesis. *Mol. Aspects Med.* **30**, 42-59.

Luberda, Z. (2005) The role of glutathione in mammalian gametes. *Reprod. Biol.* **5**, 5-17.

McGowan, A.J., Fernandes, R.S., Samali, A., and Cotter, T.G. (1996) Anti-oxidants and apoptosis. *Biochem. Soc. Trans.* **24**, 229-232.

MacWilliams, H. K. (1982) Transplantation experiments and pattern mutants in the *Dictyostelium discoideum* slug. *Symp. Soc. Dev. Biol.* **40**, 463-483.

Madeo, F., Frohlich, E., Ligr, M., Grey, M., Sigrist, S. J., Wolf, D. H., and Frohlich, K. U. (1999) Oxygen stress: a regulator of apoptosis in yeast. *J. Cell Biol.* **145**, 757-767.

Mahadeo, D. C., and Parent, C. A. (2006) Signal Relay During the Life Cycle of *Dictyostelium*. *Curr. Top. Dev. Biol.* **73**, 115-140.

Maeda, Y. (2005) Regulation of growth and differentiation in *Dictyostelium*. *Int. Rev. Cytol.* **244**, 287-332.

Maeda, M., Aubry, L., Insall, R., Gaskins, C., Devreotes, P. N., and Firtel, R. A. (1996) Seven helix chemoattractant receptors transiently stimulate mitogen-activated

protein kinase in *Dictyostelium*. Role of heterotrimeric G proteins. *J. Biol. Chem.* **271**, 3351-3354.

Maeda, Y., and Iijima, N. (1992) Cross-talks required for the acquisition of development competence in *Dictyostelium discoideum* cells. *Anim. Biol.* **1**, 145-155.

Mann, S. K., and Firtel, R. A. (1989) Two-phase regulatory pathway controls cAMP receptor-mediated expression of early genes in *Dictyostelium*. *Proc. Natl. Acad. Sci. U.S.A.* **86**, 1924-1928.

Mann, S. K., and Firtel, R. A. (1991) A developmentally regulated, putative serine/threonine protein kinase is essential for development in *Dictyostelium*. *Mech. Dev.* **35**, 89-101.

Mann, S. K., Yonemoto, W. M., Taylor, S. S., and Firtel, R. A. (1992) DdPK3, which plays essential roles during *Dictyostelium* development, encodes the catalytic subunit of cAMP-dependent protein kinase. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 10701-10705.

Mann, S. K., Brown, J. M., Briscoe, C., Parent, C., Pitt, G., Devreotes, P. N., and Firtel, R. A. (1997) Role of cAMP-dependent protein kinase in controlling aggregation and postaggregative development in *Dictyostelium*. *Dev. Biol.* **183**, 208-221.

Margolskee, J. P., and Lodish, H. F. (1980) The regulation of the synthesis of actin and two other proteins induced early in *Dictyostelium discoideum* development. *Dev. Biol.* **74**, 50-64.

McPherson, C. E., and Singleton, C. K. (1992) V4, a gene required for the transition from growth to development in *Dictyostelium discoideum*. *Dev. Biol.* **150**, 231-242.

Meister, A., and Anderson, M. E. (1983) Glutathione. *Annu. Rev. Biochem.* **52**, 711-760.

Meister, A. (1988) Glutathione. The Liver: Biology and Pathobiology, second Ed. New York: Raven Press 401-417.

Meister, A. (1994) Glutathione, ascorbate, and cellular protection. *Cancer Res. (Suppl)* **54**, 1969s-1975s.

Milne J. L., and Coukell, M. B. (1991) A Ca2+ transport system associated with the plasma membrane of *Dictyostelium discoideum* is activated by different chemoattractant receptors. *J. Cell Biol.* **112**, 103-110.

Milne J. L., and Devreotes, P. N. (1993) The surface cyclic AMP receptors, cAR1, cAR2, and cAR3, promote Ca2+ influx in *Dictyostelium discoideum* by a G alpha 2-independent mechanism. *Mol. Biol. Cell* **4**, 283-292.

Milne, J. L., Kim, J. Y., and Devreotes, P. N. (1997) Chemoattractant receptor signaling: G protein-dependent and -independent pathways. *Adv. Second Messenger Phosphoprotein Res.* **31**, 83-104.

Meredith, M. J., and Reed, D. J. (1982) Status of the mitochondrial pool of glutathione in the isolated hepatocyte. *J. Biol. Chem.* **257**, 3747-3753.

Morita, T., Amagai, A., and Maeda, Y. (2004) Translocation of the *Dictyostelium* TRAP1 homologue to mitochondria induces a novel prestarvation response. *J. Cell Sci.* **117**, 5759-5770.

Mutzel, R., Lacombe, M.L., Simon, M.N., De Gunzburg, J., and Veron, M. (1987) Cloning and cDNA sequence of the regulatory subunit of cAMP-dependent protein kinase from *Dictyostelium discoideum*. *Proc. Natl. Acad. Sci. U.S.A.* **84**, 6–10.

Newton, G. L., and Fahey, R. C. (1995) Determination of biothiols by bromobimane labeling and high-performance liquid chromatography. *Methods Enzymol.* **251**, 148-166.

Noctor, G., Mhamdi, A., Chaouch, S., Han, Y., Neukermans, J., Marquez-Garcia, B., Queval, G. and Foyer, C. H. (2012) Glutathione in plants: an integrated overview. *Plant Cell Environ.* **35**, 454-484.

Otsuka, H., and Van Haastert, P. J. (1998) A novel Myb homolog initiates *Dictyostelium* development by induction of adenylyl cyclase expression. *Genes Dev.* **12**, 1738-1748.

Pang, K. M., Lynes, M. A., and Knecht, D. A. (1999) Variables controlling the expression level of exogenous genes in *Dictyostelium*. *Plasmid* **41**, 187-197.

Parent, C. A., and Devreotes, P. N. (1996) Constitutively active adenylyl cyclase mutant requires neither G proteins nor cytosolic regulators. *J. Biol. Chem.* **271**, 18333-18336.

Pasternak, M., Lim, B., Wirtz, M., Hell, R., Cobbett, C. S., and Meyer, A. J. (2008) Restricting glutathione biosynthesis to the cytosol is sufficient for normal plant development. *Plant J.* **53**, 999-1012.

Pitt, G. S., Milona, N., Borleis, J., Lin, K. C., Reed, R. R., and Devreotes, P. N. (1992) Structurally distinct and stage-specific adenylyl cyclase genes play different roles in *Dictyostelium* development. *Cell* **69**, 305-315.

Raper, K. B. (1940) Pseudoplasmodium formation and organization in Dictyostelium discoideum. *J. Elisha Mitchell Sci. Soc.* **56**, 241-282.

Rathi, A., and Clarke, M. (1992) Expression of early developmental genes in *Dictyostelium discoideum* is initiated during exponential growth by an autocrine-dependent mechanism. *Mech. Dev.* **36**, 173-182.

Rathi, A., Kayman, S. C., and Clarke, M. (1991) Induction of gene expression in *Dictyostelium* by prestarvation factor, a factor secreted by growing cells. *Dev. Genet.* **12**, 82-87.

Reid, A.B., Kurten, R.C., McCullough, S.S., Brock, R.W., and Hinson, J.A. (2005) Mechanisms of acetaminophen-induced hepatotoxicity: role of oxidative stress and

mitochondrial permeability transition in freshly isolated mouse hepatocytes. *J. Pharmacol. Exp. Ther.* **312**, 509-516.

Sambrook, J. and Gething, M.J. (1989) Protein structure. Chaperones, paperones. *Nature* **342**, 224-225.

Saran, S., and Schaap, P. (2004) Adenylyl cyclase G is activated by an intramolecular osmosensor. *Mol. Biol. Cell* **15**, 1479-1486.

Saraste, M., Sibbald, P. R., and Wittinghofer, A. (1990). The P-loop—a common motif in ATP- and GTP-binding proteins. *Trends Biochem. Sci.* **15**, 430-434.

Sarbassov, D. D., Ali, S. M., Kim, D. H., Guertin, D. A., Latek, R. R., Erdjument-Bromage, H., Tempst, P., and Sabatini, D. M. (2004) Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. *Curr. Biol.* **14**, 1296-1302.

Saxe, C. 3rd., Ginsburg, G. T., Louis, J. M., Johnson, R., Devreotes, P. N., and Kimmel, A. R. (1993) CAR2, a prestalk cAMP receptor required for normal tip formation and late development of *Dictyostelium discoideum*. *Genes Dev.* **7**, 262-272.

Saxe, C. L. 3rd., Johnson, R., Devreotes, P. N., and Kimmel, A. R. (1991) Multiple genes for cell surface cAMP receptors in *Dictyostelium discoideum*. *Dev. Genet.* **12**, 6-13.

Schlatterer, C., Knoll, G. and Malchow, D. (1992). Intracellular calcium during chemotaxis of *Dictyostelium discoideum*: a new fura-2 derivative avoids sequestration of the indicator and allows long-term calcium measurements. *Eur. J. Cell Biol.* **58**, 172-181.

Schulkes, C., and Schaap, P. (1995) cAMP-dependent protein kinase activity is essential for preaggregative gene expression in *Dictyostelium*. *FEBS Lett.* **368**, 381-384.

Schnitzler, G.R., Briscoe, G., Brown, J.M., and Firtel, R.A. (1995) Serpentine cAMP receptors may act through a G-protein-independent pathway to induce post-aggregative development in *Dictyostelium*. *Cell* **81**, 735-745.

Segall, J. E., Kuspa, A., Shaulsky, G., Ecke, M., Maeda, M., Gaskins, C., Firtel, R. A., and Loomis, W. F. (1995) A MAP kinase necessary for receptor-mediated activation of adenylyl cyclase in *Dictyostelium*. *J. Cell Biol.* **128**, 405-413.

Shaulsky, G., and Loomis, W. F. (1995) Mitochondrial DNA replication but no nuclear DNA replication during development of *Dictyostelium*. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 5660-5663.

Schatzle, J., Bush, J., and Cardelli, J. (1992) Molecular cloning and characterization of the structural gene coding for the developmentally regulated lysosomal enzyme, α-mannosidase, in *Dictyostelium discoideum*. *J. Biol. Chem.* **267**, 4000-4007.

Sherr, C. J. (1996) Cancer cell cycles. *Science* **274**, 1672-1677.

Shi, Z. Z., Osei-Frimpong, J., Kala, G., Kala, S. V., Barrios, R. J., Habib, G. M., Lukin, D. J., Danney, C. M., Matzuk, M. M., and Lieberman, M. W. (2000) Glutathione synthesis is essential for mouse development but not for cell growth in culture. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 5101-5106.

Sies, H. (1999) Glutathione and its role in cellular functions. *Free Radical Biol. Med.* **27**, 916-921.

Simon, M. N., Pelegrini, O., Véron, M., and Kay, R. R. (1992) Mutation of protein kinase A causes heterochronic development of *Dictyostelium*. *Nature* **356**, 171-172.

Soll, D.R., Yarger, J. and Mirick, M. (1976) Stationary phase and the cell cycle of *Dictyostelium discoideum* in liquid nutrient medium. *J. Cell Sci.* **20**, 513-523.

Souza, G. M., Hirai, J., Mehta, D. P., and Freeze, H. H. (1995) Identification of two novel *Dictyostelium discoideum* cysteine proteinases that carry *N*-acetylglucosamine-1-P modification. *J. Biol. Chem.* **270**, 28938-28945.

Souza, G. M., Lu, S., and Kuspa, A. (1998) YakA, a protein kinase required for the transition from growth to development in *Dictyostelium*. *Development* **125**, 2291-2302.

Souza, G. M., da Silva, A. M., and Kuspa, A. (1999) Starvation promotes *Dictyostelium* development by relieving PufA inhibition of PKA translation through the YakA kinase pathway. *Development* **126**, 3263-3274.

Sun, T. J., and Devreotes, P. N. (1991) Gene targeting of the aggregation stage cAMP receptor cAR1 in *Dictyostelium*. *Genes Dev.* **5**, 572-582.

Sun, T. J., Van Haastert, P. J., and Devreotes, P. N. (1990) Surface cAMP receptors mediate multiple responses during development in *Dictyostelium*: evidenced by antisense mutagenesis. *J. Cell Biol.* **110**, 1549-1554.

Sussman, M. (1987) Cultivation and synchronous morphogenesis of *Dictyostelium* under controlled experimental conditions. *Methods Cell Biol.* **28**, 9-29.

Taminato, A., Bagattini, R., Gorjão, R., Chen, G., Kuspa, A., and Souza, G. M. (2002) Role for YakA, cAMP, and protein kinase A in regulation of stress responses of *Dictyostelium discoideum* cells. *Mol. Biol. Cell* **13**, 2266-2275.

Thomas, D., Klein, K., Manavathu, E., Dimmock, J. R., and Mutus, B. (1991) Glutathione levels during thermal induction of the yeast-to-mycelial transition in *Candida albicans*. *FEMS Microbiol. Lett.* **77**, 331-334.

Thomason, P. A., Traynor, D., Cavet, G., Chang, W. T., Harwood, A. J., and Kay, R. R. (1998) An intersection of the cAMP/PKA and two-component signal transduction systems in *Dictyostelium*. *EMBO J.* **17**, 2838-2845.

Townsend, D. M., Tew, K. D., and Tapiero, H. (2003) The importance of glutathione in human disease. *Biomed. Pharmacotherap.* **57**, 145-155.

Van Driessche, N., Shaw, C., Katoh, M., Morio, T., Sucgang, R., Ibarra, M., Kuwayama, H., Saito, T., Urushihara, H., Maeda, M., Takeuchi, I., Ochiai, H., Eaton, W., Tollett, J., Halter, J., Kuspa, A., Tanaka, Y., and Shaulsky, G. (2002) A

transcriptional profile of multicellular development in *Dictyostelium discoideum*. *Development* **129**, 1543-1552.

van Es, S., Virdy, K. J., Pitt, G. S., Meima, M., Sands, T. W., Devreotes, P. N., Cotter, D. A., and Schaap, P. (1996) Adenylyl cyclase G, an osmosensor controlling germination of *Dictyostelium* spores. *J. Biol. Chem.* **271**, 23623-23625.

Van Haastert P. J. (1995) Transduction of the chemotactic cAMP signal across the plasma membrane of *Dictyostelium* cells, *Experientia* **51**, 1144-1154.

Van Lookeren Campagne, M. M., Franke, J., and Kessin, R. H. (1991) Functional cloning of a *Dictyostelium discoideum* cDNA encoding GMP synthetase. *J. Biol. Chem.* **266**, 16448-16452.

Vernoux, T., Wilson, R. C., Seeley, K. A., Reichheld, J. P., Muroy, S., Brown, S., Maughan, S. C., Cobbett, C. S., Van Montagu, M., Inze, D., May, M. J., and Sung, Z. R. (2000) The ROOT MERISTEMLESS1/CADMIUM SENSITIVE2 gene defines a glutathione-dependent pathway involved in initiation and maintenance of cell division during postembryonic root development. *Plant Cell* **12**, 97-109.

Viña, J., Saez, G. T., Wiggins, D., Roberts, A. F., Hems, R., and Krebs, H. A. (1983) The effect of cysteine oxidation on isolated hepatocytes. *Biochem. J.* **212**, 39-44.

Wang, B., and Kuspa, A. (2002) CulB, a putative ubiquitin ligase subunit, regulates prestalk cell differentiation and morphogenesis in *Dictyostelium spp*. *Eukaryot. Cell* **1**, 126-136.

Weeks, G., and Weijer, C. J. (1994) The *Dictyostelium* cell cycle and its relationship to differentiation. *FEMS Microbial. Lett.* **124**, 123-130.

Wharton, R. P., Sonoda, J., Lee, T., Patterson, M., and Murata, Y. (1998) The Pumilio RNA-binding domain is also a translational regulator. *Mol. Cell* **1**, 863-872.

Winkler, A., Njålsson, R., Carlsson, K., Elgadi, A., Rozell, B., Abraham, L., Ercal, N., Shi, Z. Z., Lieberman, M. W., Larsson, A., and Norgren, S. (2011) Glutathione

is essential for early embryogenesis – Analysis of a glutathione synthetase knockout mouse. *Biochem. Biophys. Res. Commun.* **412**, 121-126.

Williams, J. (1995) Morphogenesis in *Dictyostelium*: new twists to a not-so-old tale. *Curr. Opin. Genet. Dev.* **5**, 426-431.

Wu, L., Hansen, D., Franke, J., Kessin, R. H., and Podgorski, G. J. (1995) Regulation of *Dictyostelium* early development genes in signal transduction mutants. *Dev. Biol.* **171**, 149-158.

Wu, L., Valkema, R., Van Haastert, P. J., and Devreotes, P. N. (1995) The G protein beta subunit is essential for multiple responses to chemoattractants in Dictyostelium. *J.Cell Biol.* **129**, 1667-1675.

Wu, Y., Zhang, X., Bardag-Gorce, F., Robel, R. C., Aguilo, J., Chen, L., Zeng, Y., Hwang, K., French, S. W., Lu, S. C., and Wan, Y. J. (2004) Retinoid X receptor α regulates glutathione homeostasis and xenobiotic detoxification processes in mouse liver. *Mol. Pharmacol.* **65**, 550-557.

Yuen, I. S., Jain, R., Bishop, J. D., Lindsey, D. F., Deery, W. J., Van Haastert, P. J., and Gomer, R. H. (1995) A density-sensing factor regulates signal transduction in *Dictyostelium*. *J. Cell Biol.* **129**, 1251-1262.

Zamore, P. D., Bartel, D. P., Lehmann, R., and Williamson, J. R. (1999) The PUMILIO-RNA interaction: a single RNA-binding domain monomer recognizes a bipartite target sequence. *Biochemistry* **38**, 596-604.

Zhang, H., and Forman, H. J. (2012) Glutathione synthesis and its role in redox signaling. *Semin. Cell Dev. Biol.* **23**, 722-728

Zhang, B., Gallegos, M., Puoti, A., Durkin, E., Fields, S., Kimble, J., and Wickens, M. P. (1997) A conserved RNA-binding protein that regulates sexual fates in the *C. elegans* hermaphrodite germ line. *Nature* **390**, 477-484.

Zhang, N., Long, Y., and Devreotes, P. N. (2001) Gγ in *Dictyostelium*: its role in localization of Gβγ to the membrane is required for chemotaxis in shallow gradients. *Mol. Biol. Cell* **12**, 3204-3213.

국문초록

Glutathione은 진핵생물의 세포 내에서 높은 농도로 존재하는 tripeptide로 –SH(thiol) 작용기를 가지고 있어 중요한 세포 내부의 반응에 참여한다. 선행연구결과에 따르면, glutathione은 세포의 성장과 분화 모두에 있어 중요한 작용을 한다. Glutathione을 합성할 수 없는 균주에서 세포 내 methylglyoxal의 축적으로 인한 세포의 성장저해와 세포사멸이 관찰되었고, 분화에 있어서도 glutathione없을 때에는 초기배아발생에 치명적인 결함을 유발해 배아의 정상적인 발생을 저해하는 것이 보고되어있다. Glutathione의 주요성은 많이 보고되고 있지만 정확한 작용기작에 대한 이해는 부족하다. 본 연구는 뚜렷하고 관찰하기 쉬운 분화형태를 가지고 있는 *Dictyostelium discoideum*을 이용하여, 세포 내 glutathione이 완전하게 제거되었을 때 분화에 어떤 영향을 미치는지를 확인하여 분화과정에서 glutathione의 역할을 규명하고자 하였다.

Glutathione을 합성할 수 없는 돌연변이주(GCS 결실균주)는 외부에서 glutathione을 추가적으로 넣어주지 않으면 분화를 시작하지 못하였다. 영양분이 고갈되면 *Dictyostelium*은 세포 외부의 cAMP의 농도를 인지하고 한 방향으로 모여들어 다세포의 군집형태를 이루는데, GCS 결실균주는 군집형태를 이루지 못하였다. GCS 결실균주의 분화양상은 액체 분화배지에서 분화를 유도시켜 더욱 자세히 관찰되었다. 야생균주는 세포들이 모여 하나의 세포군집을 이루었지만, GCS 결실균주는 계속해서 단일 세포의 상태로 존재하고 있었다. 이런 GCS 결실균주의 분화과정에서의 결함은 –thiol 작용기를 가지고 있는 다른 화학물질(dithiothreitol (DTT), *N*-

acetylcysteine (NAC))과 일반적인 항산화물질로 알려져 있는 ascorbic acid을 첨가해주어도 회복되지 않았다. 즉, *Dictyostelium*의 분과과정에서 glutathione은 세포내 산화·환원 환경을 조절하는 것뿐만 아니라 고유의 역할을 하고 있음을 알수 있었다.

*Dictyostelium*의 분화는 많은 유전자들의 발현을 정밀하게 조절하여 적절하게 일어나는 일련의 과정들이다. 분화가 시작되면, 세포성장에 관여하던 유전자(*cprD*)들의 발현은 감소되고 분화에 필요한 유전자들의 발현은 크게 증가한다. 그 중에서도 cAMP신호전달에 관여하는 단백질 유전자들인 *carA*와 *acaA*의 발현은 분화초기단계 조절에 중요한 역할을 한다. GCS 결실균주에서는 *cprD*의 발현이 증가되어 있고, *carA*와 *acaA*의 발현이 현저하게 감소되어있었다. 외부에서 cAMP를 첨가해 자극해주거나 cAMP를 인식하는 단백질(cAR1)을 과량발현시켜 cAMP 신호전달을 인위적으로 유도하여도 glutathione의 첨가 없이는 분화하지 못하였다. 즉, *Dictyostelium*의 분화에서 glutathione은 cAMP에의한 신호전달체계가 작용하는 시기보다 더 앞선 단계에 작용할 것으로 생각된다.

GCS 결실균주에서는 YakA의 유전자가 발현되지 않았다. 또한 *yakA*의 발현양은 세포 내부의 glutathione의 농도에 비례하여 증가하는 것이 관찰되었다. 그리고 분화형태나 분화초기에 중요한 역할을 하는 것으로 알려져 있는 유전자들의 발현양상이 YakA 결실균주와 GCS 결실균주에서 유사하였다. YakA를 GCS 결실균주에 과량발현시키면, glutathione이 없어도 분화하여 다세포성 세포군집을 형성하였고, 분화초기에 관여하는 것으로 알려져있는 유전자들또한 정상적으로 발현되었다. GCS 결실균주의 분화결함은 YakA를 발현시켜줌으써 회복되었지만, YakA 결실균주는 glutathione을

첨가해주어도 분화를 하지 못하였다. 또한 YakA 과량발현에 의해 세포 내 glutathione 농도나 *gcsA*의 발현양이 크게 영향을 받지 않는 것으로 보아 YakA가 세포 내 glutathione의 농도를 직접적으로 조절하는 것은 아닌 것으로 생각된다.

위의 결과를 종합하여 보았을 때, 세포 내 glutathione은 영양분의 고갈과 같은 분화 환경조건에 반응하여 YakA 유전자의 발현을 유도하고, 그 하위단계 작용단백질들의 발현을 조절하여 YakA 신호전달을 활성화 시킴으로써 *Dictyostelium*의 세포성장에서 분화로의 전환을 조절하는 것으로 생각된다.

주요어; Glutathione, YakA, 분화, *Dictyostelium discoideum*